UV INDUCED DNA DAMAGE AND REPAIR IN MARINE BACTERIA

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CERTIFICATE

This is to certify that the project report entitled **"UV induced DNA damage and repair in marine bacteria"** submitted by **Ms. Sonam Ganeriwal** to the Department of Life Science, National Institute of Technology, Rourkela in partial fulfillment of the requirements for the degree of Masters of Science in **LIFE SCIENCE** is a bonafide record of work carried out by her under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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I bow my head before the Almighty for his blessings on me.

Declaration

I hereby declare that the thesis entitled **"UV induced DNA damage and Repair in Marine bacteria"** submitted to Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the requirements for the degree of master of science in life science is an original piece of research work done by me under the guidance of Dr. Surajit Das, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. No part of this work has been done by any other research person and has not been submitted for any other purpose.

Sonam Ganeriwal

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LIST OF SYMBOLS AND ABBREVIATION USED

+	Positive
-	Negative
h	Hour
min	Minute
sec	Second
UV	Ultraviolet
µl	Microlitre
rpm	Revolution per minute
CFU	Colony forming unit
PBS	Phosphate Buffer saline
◦C	Degree Celsius
SD	Standard deviation
µm	Micrometer
ml	Mililitre

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The continuous depletion of the Earth's ozone layer by anthropogenic activities has fueled concern about the impact of increasing solar ultraviolet-B radiation (UV-B) on aquatic ecosystems. The DNA is one of the key targets for UV-induced damage in the aquatic organisms. UV radiation induces two of the most abundant mutagenic and cytotoxic DNA lesions, cyclobutane pyrimidine dimers (CPDs) and pyrimidine pyrimidone photoproducts (6-4PPs) and their Dewar valence isomers. Thus, the aquatic organisms have developed a number of enzymes, repair and tolerance mechanisms to counteract the damaging effects of UV. The continuing ozone depletion not only caused DNA damage but also causes an abrupt collapse of primary photosynthetic production, resulting in subtle, community-level responses that could ultimately impact on higher trophic levels. The pH of oceans is also changing due to increase in amount of dissolved CO_2 thus causing ocean acidification and disturbing the biogeochemical cycles in the marine environment. To study the consequences of ocean acidification, the response of marine bacteria (in terms of survival) under pH changes upon UVR exposure, preliminary work has been done on marine bacteria *Pseudomonas pseudoalcaligenes* NP103 and *P. aeruginosa* N6P6 isolated from Odisha coast. The sensitivity of the bacterium to UV-B under different dosages has been determined by colony counting and spectrophotometry growth method. The percentage survival calculated by colony counting method was about 0.005% and 0.004% for *P. pseudoalcaligenes* NP103 and *P. aeruginosa* N6P6 respectively (UVR exposure of 5 sec). The survival upon exposure to different UV dosage was studied spectrophotometrically. In case of *P. pseudoalcaligenes* NP103 the survival was maximum at pH 8 which decreased with decline in pH of the medium. Whereas in *P. aeruginosa* N6P6, optimum growth and survival was observed at pH 7. The preliminary findings suggest that pH has a crucial role in DNA repair system and response varies with bacterial species. Decline in ocean water pH from current pH 8.2-8.4 is expected to affect the survival of marine bacteria upon UV damage in some bacterial groups.

KEYWORDS: Ultraviolet-B radiation, Primary photosynthetic production, CPD, 6-4PP, ocean acidification.

1. INTRODUCTION

Earths climatic conditions are changing drastically due to environmental pollution leading to global warming. Global warming is the unequivocal and continuing to increase the average temperature of earth. Since 1971, 90% of this warming has occurred in the oceans. Oceans play a dominant role in energy storage, the term global warming is also used to refer to increases in average temperature of the air and sea and even at Earth's surface. From the early 20th century, the global sea surface temperature has increased about 0.8 °C with about two-thirds of the increase occurring since last 2 decades (Bertrand and Ypersele., 2002). There is conclusive evidence that the stratospheric ozone layer, which protects the Earth from the biologically most hazardous short-wavelength solar radiation, is currently experiencing continuous depletion. It is catalyzed by anthropogenically released atmospheric pollutants such as chlorofluorocarbons (CFCs) chlorocarbons (CCs) and organo-bromides (OBs). Thus a high proportion of UV light is able to reach the earth atmosphere causing deleterious effects.

Ozone depletion has been reported in the Antarctic, the Arctic and subarctic regions, but it is most widespread over the Antarctic where ozone levels have declined by more than 70% during late winter and early spring during the last few decades. This decline in ozone level is commonly attributed to polar vortex, a combination of extreme cold and stratospheric circulation that results in conditions that are favorable for the reaction of CFC and ozone. Polar stratospheric clouds play an important role in the formation of the springtime Antarctic ozone hole by activating chlorine and denitrifying the stratosphere (Wallace *et al.*, 2000). Ozone depletion and the associated increased UV radiation have been predicted to continue throughout most of the century. Higher UVR levels have also been shown to disrupt aquatic food webs and reduce the biological sinking capacity of aquatic environments for atmospheric CO_2.The xenotoxic effects

of solar UV radiation have precluded the development of terrestrial life for two to three billion years, before the stratospheric ozone layer even developed. When the primitive life appeared on the early earth, there was no ozone layer to protect these primitive organisms from solar UV radiations. Thus it is generally assumed that life originated in places that were shielded from UV light, like hydrothermal vents deep under ocean. Later as the protecting ozone layer expanded in the atmosphere, other zones of the planet became habitable and the different life forms spread over the planet (Ward and Brownlee., 2003). Ultraviolet radiation induces harmful effects in all living organisms ranging from prokaryotes to eukaryotic lower and higher plants, animals and humans. While UV-C (<280 nm) radiation is ecologically irrelevant since it is absorbed by oxygen and ozone in the Earth's atmosphere, the longer wavelength UV-B (280–315 nm) and UV-A (315–400 nm) radiation can have significant effects on the biotic community, even though the majority of the extraterrestrial UV-B is absorbed by stratospheric ozone (Zenoff *et al.,* 2006).

The adverse effects of solar radiation on living systems are mostly attributed to the small amount of UV-B that is absorbed by cellular DNA. As DNA is the deposit bank of genetic information in every living cell, its integrity and stability are essential for life. But DNA is not inert rather it is a chemical entity subjected to assault from the environment, and if any resulting damage remains unrepaired, it can lead to mutation. The vast majority of DNA damage affects the primary structure of the double helix by chemically modifying the bases. These modifications can in turn disrupt the molecules regular double helical structure by introducing non-native chemical bonds or bulky adducts that do not fit in the standard double helix. DNA usually lacks tertiary structure unlike RNA and protein and thus there is no damage or disturbance at that level (Sinha and Hader., 2002).

The accurate transmission of genetic information from one cell to its daughters is the key for the survival of organisms. Such faithful transmission requires extreme accuracy in replication of DNA and precision in chromosome distribution. It also requires the ability to survive such spontaneous and induced DNA damage by minimizing the occurrence of heritable mutations. To achieve this goal organisms have developed efficient DNA repair mechanisms or DNA damage response in order to counteract the lethal effects of DNA lesions (Zhou and Elledge., 2000). A number of important repair pathways, which cope with different kinds of environmental mutations are present. Photoreactivation which works with the help of photolyase enzyme in presence of light base excision repair that replaces damaged bases in the DNA code, nucleotide excision repair which replaces a string of bases if one or more is damaged. NHEJ: non-homologous end joining that fixes double-strand breaks in the DNA double-helix. Homologous repair which fixes double-strand breaks in and inter strand cross-links in DNA. Mismatch repair that corrects mismatches in the sequence of base and SOS repair which acts when other repair mechanism fails. These repair mechanism work efficiently in both prokaryotes as well as eukaryotes.

But in contrast to the terrestrial environment where the primary producers are higher plants, which are generally very large and survive for many months or years, in the oceans the primary producers are microbes that lives only for only a day or so. Life in the sea is thus reliable on marine microbes. These microbial plants like phytoplankton and cyanobacteria are extremely important for the planet since they produce about 50% of the total oxygen that is produced globally by photosynthesis each year. Bacteria and archaea are very important in maintaining the productivity of the oceans by recycling nutrients that are required by the phytoplankton. Bacteria play an important role in biogeochemical cycling of many elements.

Ocean productivity depends on a wide range of microbial species, a diversity that has only recently been revealed through the sequencing of DNA from the oceans. There is still a very poor understanding of the microbes that are responsible for these fundamentally important biogeochemical cycles that maintain the productivity of the oceans. The truth is that we can more readily chemically measure the result of microbial activity, such as release of greenhouse gases like methane or nitrous oxide to the atmosphere, than we can identify and describe the organisms involved in the process.

Marine bacteria accounts up to 90% of the cellular DNA in oceanic environment (Joux *et al.,* 1999). These microbes play an important role in nutrient cycling in aquatic ecosystems and form a fundamental link in the carbon transfer process popularly known as the microbial loop. At the begining of most pelagic food webs, the nano- and picoplankton (0.2–20 μm) are believed to process 14–80% of primary production by solublizing particulate organic matter by mediating geochemical cycling of important minerals like sulfur, phosphorus, and nitrogen, functioning as mineralizers and by acting as secondary producers which in turn are consumed by higher trophic levels. Generally dissolved organic carbon (DOC) is introduced into the marine environment from lysis of bacterial cell, sudden cell senescence, sloppy feeding by zooplankton, the waste products or excreta produced by aquatic animals, and the breakdown and dissolution of organic particles from terrestrial plants . Bacteria in the microbial loop decompose this particulate detritus to utilize this energy-rich matter for growth. Since more than 95% of organic matter in marine ecosystems consists of high molecular weight polymeric compounds like protein, polysaccharides, lipids, only a small portion of total dissolved organic matter (DOM) is readily utilizable to most marine organisms at higher trophic levels. This means that dissolved organic carbon is not available directly to most marine organisms; marine bacteria

introduce this organic carbon into the food web, resulting in additional energy becoming available to higher trophic levels. The marine bacteria is of particular importance in increasing the efficiency of the marine food web by utilizing the dissolved organic matter (DOM), which is mostly unavailable to most marine organisms, thus it aids in recycling of organic matter and nutrients and mediates the transfer of energy above the thermocline.

More than 30% of dissolved organic carbon (DOC) incorporated into bacteria is respired and released as carbon dioxide (Azam and Malfatti., 2007). The marine bacteria in the water column accelerate mineralization through regenerating production in nutrient-limited environments (e.g. oligotrophic waters). Marine bacteria are the base of the food web in most oceanic environments, and they improve the trophic efficiency of both marine food webs and important aquatic processes such as the productivity of fisheries and the amount of carbon exported to the ocean floor. Thus, the microbial loop, together with primary production, controls the productivity of marine systems in the ocean. Marine bacteria help in tracing gas production and metal availability. Microbes drive the production and consumption of many other potent climate-active gases – methane, nitrous oxide, dimethylsulfide and organohalides. They have very important implications for potential climate-feedback mechanism (Fig 1).

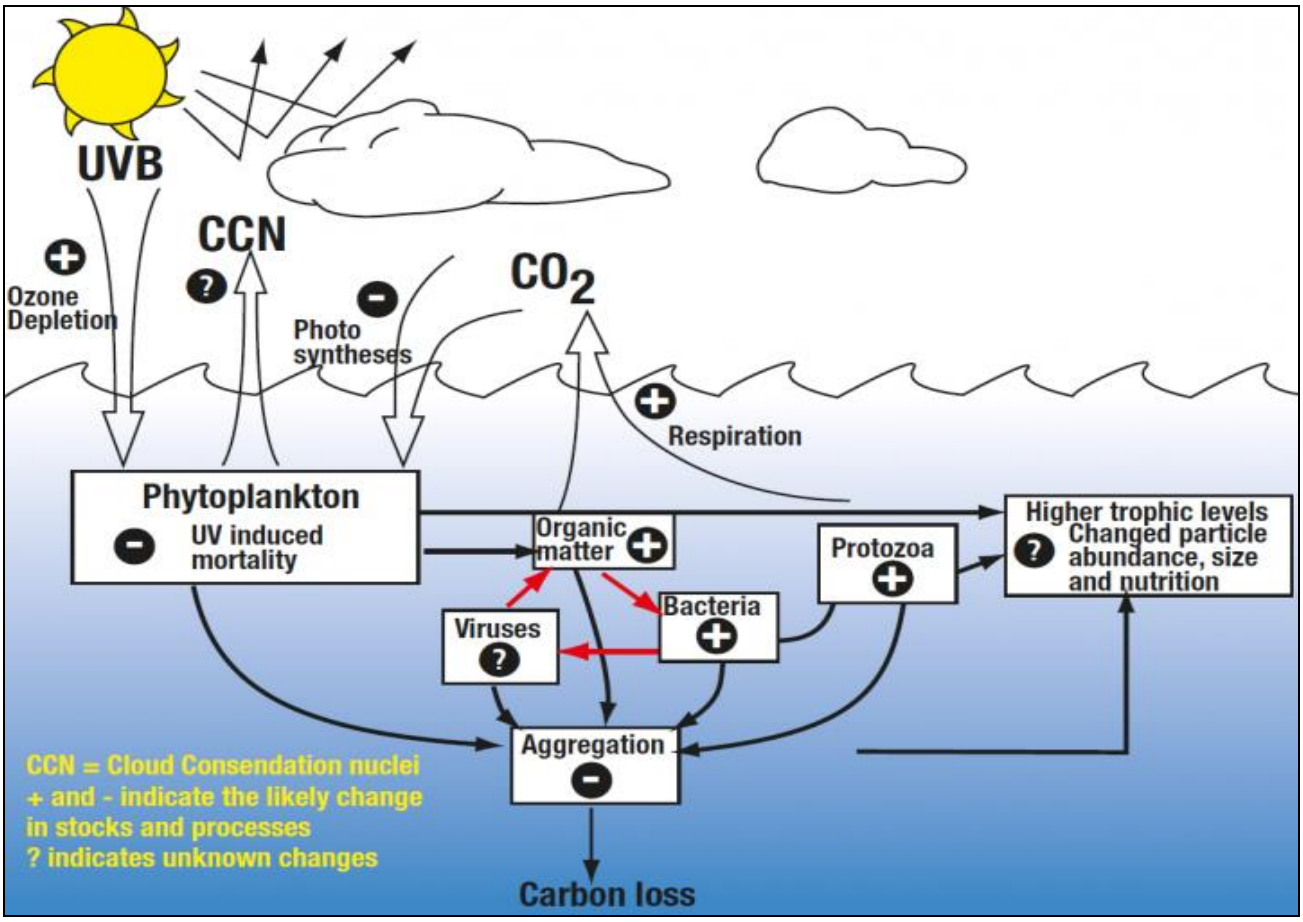


Fig 1. Schematic diagram indicates the result of ozone depletion on marine microbes and the resulting impact on global climate, Australian Antartic magazine, Issue 1, 2001.

DNA is thus prone to many damages and produce various damage products such as SSB (single strand break), DSB (double strand break), CPDs (cyclobutane pyrimidine dimers), 6-4PPs (6-4 photoproducts) and their Dewar valence isomers several DNA detection methods such as PCR (polymerase chain reaction),comet assay, TUNEL (Terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) assay, HPLC-Electrospray tandem mass spectrometry, FISH (Fluorescence in situ hybridization),Gas chromatography-mass spectrometry and electrochemical methods, are commonly used to detect DNA damage in various organisms (Kumari *et al.,* 2010).There is a vast correlation between ocean acidification,

increased UVR and anthropogenic pollutants in marine environments. Microbes are mediator of major biogeochemical cycles, providing fundamental ecosystems services such as environmental detoxification and recovery. Therefore it is important to understand how predicted changes to oceanic salt concentration pH and UVR, will affect microbial pollutant detoxification processes in marine ecosystems. The effect of UV on these marine microbes is highly deleterious and can cause heavy damage to the marine environment as these marine microbes have no functional redundancy have simple haploid genome and no protective pigmentation unlike the eukaryotes.

2. REVIEW OF LITERATURE

2.1 DNA DAMAGE

The change in the regular structure of DNA double helix by chemically modifying bases is called as DNA damage. DNA damage is distinctly different from mutation, although both are types of error in DNA; former is an abnormal chemical structure in DNA, whereas mutation is a change in the sequence of standard nucleotide base pairs.DNA damage can be grouped into 2 ways:

1. *Endogeneous DNA damage:* It is caused due to attack by reactive oxygen species (ROS) produced from normal metabolic byproducts or by spontaneous mutation, like the process of oxidative deamination and replication errors. The replication of damaged DNA prior to cell division can lead to incorporation of wrong bases corresponding the damaged ones. Progeny cells that inherit such wrong bases lead to mutations from which the recovery of original DNA sequence is impossible. Due to endogeneous DNA damage 5 main types of damage products are formed .They are oxidation of bases e.g. 8-oxo-7,8-dihydroguanine,commonly known as (8-oxoG) and generation of DNA strand interruptions from reactive oxygen species, alkylation of bases such as methylation, and formation of 7-methylguanine, 1-methyladenine and 6-O-Methylguanine. (Sultana *et al.*, 2010). Hydrolysis of bases takes place such as deamination, depurination and depyrimidination. Bulky adduct formation, benzo[a]pyrenediol, epoxide-dG adduct and aristolactam I-dAadduct. Mismatch of bases occur due to errors in DNA replication, in which the wrong DNA base is sewed into place in a newly forming DNA strand.

2. Exogeneous DNA damage: It is caused by external agents such as ultraviolet [UV 200-400 nm] radiation from the sun other electromagnetic rays, including x-rays and gamma rays, hydrolysis or thermal disruption, and mainly man-made mutagenic chemicals, especially aromatic compounds which act as DNA intercalating agents .Damage caused by exogenous agents comes in varied forms. They are generally pyrimidine dimers. UV-B light causes crosslinking between adjacent cytosine and thymine bases creating these dimers. This is called direct DNA damage. UV-A light creates free radicals. The damage caused by free radicals is called indirect DNA damage. Ionizing radiation created by radioactive decay creates breaks in DNA strands. Low-level ionizing radiation may also induce irreparable DNA damage leading to replicational and transcriptional errors. Thermal disruption at elevated temperature increases the rate of depurination and single-strand breaks. Hydrolytic depurination is seen in the thermophilic bacteria, growing in hot springs at 40-80 °C. Industrial chemicals such as vinyl chloride and hydrogen peroxide, and chemicals such as polycyclic aromatic hydrocarbons (PAHs)found in smoke, soot and tar create a massive diversity of DNA adducts like ethenobases, oxidized bases, alkylated phosphotriesters and even causes crosslinking of DNA strands.

2.2 DNA DAMAGE PRODUCTS

As a result of deleterious effect of UV light, many DNA damage products are formed. They are:

- Cyclobutane pyrimidine dimmers
- 6,4 Photoproducts
- Dewar valence isomers
- Spore photoproduct

- Single strand breaks
- 8 0xo-G

2.2.1 CYCLOBUTANE PYRIMIDINE DIMERS

They constitute the major DNA photoproduct upon exposure to UV-B light. They arise from a 2+2 cycloaddition of the C5–C6 double bond of adjacent pyrimidine bases (Fig. 2). Six diastereomers may be generated from thymine depending on the position of the pyrimidine moieties with respect to the cyclobutane ring and on the relative orientation of the two C5-C6 bonds. Due to steric constraints only syn isomers can be generated within DNA and oligonucleotides. The cis-syn form is produced in large excess in respect to trans-syn diastereomers. The later DNA photoproducts are present only within single stranded and denatured DNA (Ravanat *et al.*, 2001). These have been characterized extensively by several spectroscopic techniques such as IR, UV and NMR with mass spectrometry measurement. Its formation could be reversed through UV-C irradiation by photo induced splitting of cyclobutane ring. But CPD exhibit a residual absorption at 254 nm. This allows their photoreversion into the starting pyrimidine bases or into Uracil when cytosine containing dimer undergo deamination as a result of hydrolytic substitution of the C4 amino acid by an OH group.

Deamination may be involved in mutagenesis since the presence of Uracil containing photoproducts induces the predominant incorporation of adenine at the site of Uracil. Moreover photoreversion by photolyase repair enzyme in some organism is expected to lead to the release of a Uracil residue in place of Cytosine.

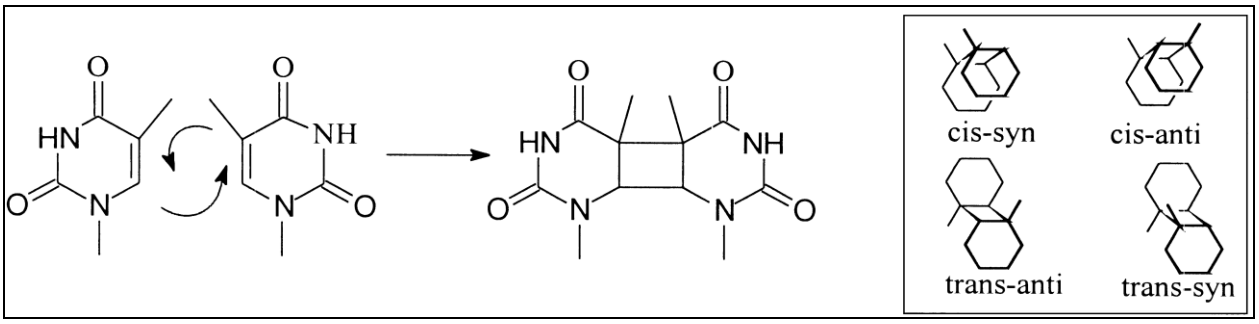


Fig 2. Formation of thymine cyclobutane dimmers (adapted from Ravanat *et al.*, 2001)

2.2.2 6-4 PHOTOPRODUCTS AND DEWAR VALENCE ISOMERS

The formation of 6-4 photoproducts involves a single excited state. They arise from a 2+2 cyclo addition involving the C5- C6 double bond of the 5'end pyrimidine and the C4 carbonyl group of the 3'end thymine. It leads to formation of an unstable oxetane or azetidine (Fig. 3). Spontaneous rearrangement of the oxetane or azitidine give rise to 6, 4 photoproducts. They occur at 1/3rd of frequency of CPDs but are more mutagenic in comparison to them (Ravanat *et al.*, 2001). Tranlesion polymerase frequently forms this type of DNA lesions. However dewar valence isomer has low mutagenic potential and produces a broad range of mutation. It is much more unstable then the 6,4photoproducts.

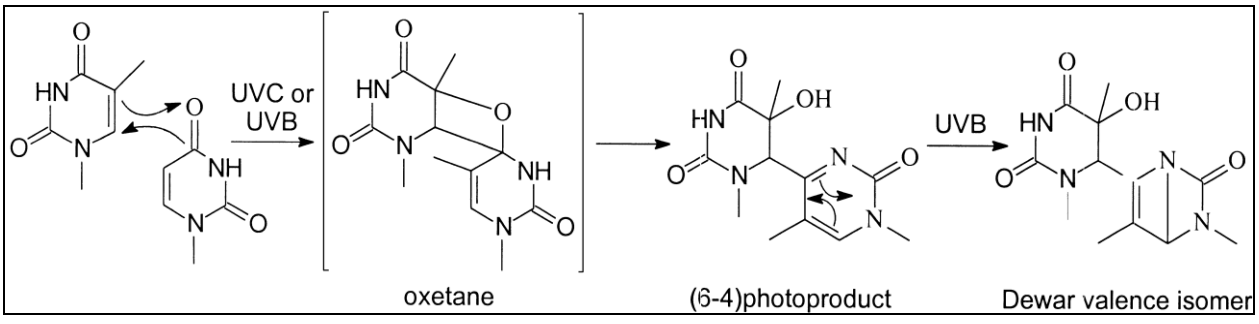


Fig 3. Formation and photoisomerization of the thymine (6-4) photoproduct (adapted from Ravanat *et al.*, 2001)

8 OXOGUANINE:

It is one of the most common DNA lesions resulting from reactive oxygen species and results in a mismatched pairing with Adenine resulting in G to T and A to C substitutions in the genome. It is repaired by the DNA glycosylase which is encoded by the gene OGG1. It is found in prokaryotes, eukaryotes and even archae. It helps in base excision repair.OGG1 is a bifunctional glycosylase, as it is able to cleave both ,the glycosidic bond of the mutagenic lesion and cause a strand break in the DNA backbone (Dizdaroglu and Vos., 2005).

2.3 DNA REPAIR MECHANISM

DNA repair is a collection of processes by which a cell identifies and corrects damage to the DNA molecules. The DNA repairing ability of a cell is vital for maintaining the integrity and normal functioning of the organism. DNA lesions may cause structural damage to the DNA molecule by altering or eliminating the cell's ability to transcribe the gene coded by the affected DNA. The rate of DNA repair depends on various factors such as the cell type, age of the cell, and extracellular environment. If a cell has accumulated a large amount of DNA damage, or it can no longer effectively repair the induced damage it enter one of three possible states such as senescence, the complete state of dormancy which is irreversible ,cell suicide commonly known as apoptosis or continuous cell division. The transmission of genetic information from parent cell to its daughter cells with accuracy is the key for the survival of every organisms. Such faithful and accurate transmission requires extreme perfection in DNA replication and precision in chromosome distribution. It also requires the ability to withstand spontaneous and induced DNA damage by minimizing the number of heritable mutations. To achieve this goal organisms have developed efficient DNA repair mechanisms in order to counteract the lethal effects of DNA damage. Specialized repair proteins scan the genome perfectly for the presence of DNA lesions.

Once a lesion recognition protein encounters a mismatched base, an apurinic or an apyrimidinic site and structurally altered bases, it stimulates an efficient DNA repair mechanism, which ultimately leads to the remodelling of the perfectly double stranded DNA with complete genetic information. DNA damage induced by UV radiation is dependent on wavelength: UV-A (320 nm to 400 nm) causes only indirect damage to DNA, lipids and proteins by forming reactive oxygen intermediates. On the other hand, UV-B (280 - 320 nm) and UV-C (100 - 280 nm) causes both direct and indirect damage because of the strong absorption at wavelengths below 320 nm by the DNA molecule. Bacteria have numerous repair mechanisms in response to UV induced damage. These repair mechanisms are basically of 2 types - dark repair (DR) and photoreactivation .which are further classified into different mechanism.

2.3.1 PHOTOREACTIVATION

Photoreactivation is one of the simplest and ancient repair systems involving the use of a single enzyme photolyase. Since DNA photolyases are found in numerous archaebacteria, they are considered to be ancient repair proteins, which may have helped in the evolution of the earliest organisms on primitive Earth. To remove DNA lesions formed by UV, many organisms contain the photolyase enzyme that binds to CPDs specifically and reverses the damage using the energy of light process known as photoreactivation. CPD photolyases are said to be reported in bacteria, fungi, plants, invertebrates as well as many vertebrates, while 6–4 photolyases have been identified in *Drosophila*, silkworm, and *Xenopus laevis*, but are absent in *E. coli* and yeast (Thoma and Hanawalt., 1999). Photolyases are said to be absent or non-functional in humans. DNA photolyases are monomeric flavin-dependent repair enzymes having a molecular weight of 50 to 65 kDa. Ten to twenty enzyme molecules scans the genome effectively for UV lesions in every cell nucleus. DNA photolyases have two chromophores.

One of the chromophores which can be either 5, 10-methenyltetrahydrofolate or 8-hydroxy-5 deazariboflavin, having absorption maxima of 380and 440 nm respectively. It is a light-harvesting antenna that absorbs the blue-light photon and transfers excitation energy to the active catalytic cofactor, which is invariably a two electron-less flavin–adenine dinucleotide (FADH). In the excited state flavin then donates an electron to the CPD which splits the cyclobutane ring, transferring the electron back to the flavin concomitantly and two canonical bases are generated. CPD photolyases recognize CPDs with a selectivity similar to that of sequence specific DNA-binding proteins, which suggests that they could compete with histones for DNA accessibility in a manner similar to transcription factors. When photolyase binds to a CPD, the efficiency of photoreactivation becomes extremely high. For every blue-light photon absorbed one dimer gets splitted. Photolyase genes have been cloned from a number of microbes like bacteria and fungi and their sequences display similar homologies. In *E. coli* the *phr* gene is found to be coding for deoxy ribo dipyrimidine photolyase that binds in the dark to the thymine dimer along with the cofactor folic acid. When the cell is then exposed to light, folic acid absorbs a photon and uses the energy to break the cyclobutane ring of the thymine dimer. The photolyase then leaves the DNA creating an undamaged strand.

Although the light-driven splitting mechanism of photoreactivation is easily understood but a number of aspects of the repair process remain uncertain such as how repair enzymes recognize single DNA lesions with extreme high precision in megabase prokaryote which is structurally heterogeneous how the initial reduction of FAD to $FADH^-$ takes place based on site-directed mutagenesis. It was suggested that the formation of $FADH^-$ results from a temporary photo-reduction. It requires an electron transfer from a far away tryptophan to the light-excited FAD radical quartet state. In addition, by using time-resolved absorption spectroscopy it has

been shown recently in *E. coli* DNA photolyase that the excited FAD radical withdraws an electron from a nearby tryptophan in 30 ps (Aubert *et al.,* 2000). After subsequent electron transfer along a chain of three tryptophans, the most remote tryptophan releases a proton to the solvent in about 300 ns, showing that electron transfer occurs before proton dissociation. A similar process may take place in photolyase-like blue-light receptors, how the enzymes mediate the energy and electron transfer processes in order to achieve repair with almost maximal efficiency (quantum yield= 0.7–0.9), and in view of the lack of any knowledge of how photolyases recognize their substrate, the different cleavage rates observed for dimers possessing different configurations and constitutions remain uncertain.

2.3.2 EXCISION REPAIR

In contrast to light repair mechanism, dark repair pathways are much more complex and tedious. They do not directly reverse DNA damage but replace the damaged DNA with new, undamaged nucleotides forming a completely new DNA strand. Basically there are two major categories of excision repair pathways: Base Excision Repair (BER) and Nucleotide Excision Repair (NER).

2.3.2.1 BASE EXCISION REPAIR

The base excision repair pathway has specifically evolved to protect the cells from the deleterious effects of endogenous DNA damage which is induced by hydrolysis, reactive oxygen species (ROS) or other intracellular metabolites that modify the double helical DNA base structure. In addition, BER is also efficient in withstanding lesions produced by strong alkylating agents and ionizing radiations such as X-rays, Gamma rays etc which are similar to those induced by endogenous factors. The basic enzymes involved in BER are DNA glycosylases, which remove different types of modified and damaged bases by cleaving the *N*-glycosidic bond

between the base and the 2- deoxyribose moieties of the nucleotide residues (Rastogi *et al.*, 2010).

Different kinds of damages are removed by different types of DNA glycolases, and the specificity of the repair pathway is determined by the type of glycosylase involved .Once the base is removed, the apurinic/ apyrimidinic (AP) site produced is removed by an AP endonuclease or an AP lyase. It nicks the DNA strand 5'or 3' to the AP site, respectively. The remaining deoxyribose phosphate residue is removed by a phosphodiesterase enzyme and the resulting gap is filled by DNA polymerase and then the strand is sealed by DNA ligase (Fig 4). It has been suggested that the repair polymerase pol β itself posses the ability to excise the 5'deoxyribose phosphate residue which is generated by the combined action of DNA glycosylases and class II AP endonucleases. The polβ enzyme achieves the incorporation of a single nucleotide after excision of the damaged base which is generally a short patch. A long patch repair pathway may also be involved in the BER.

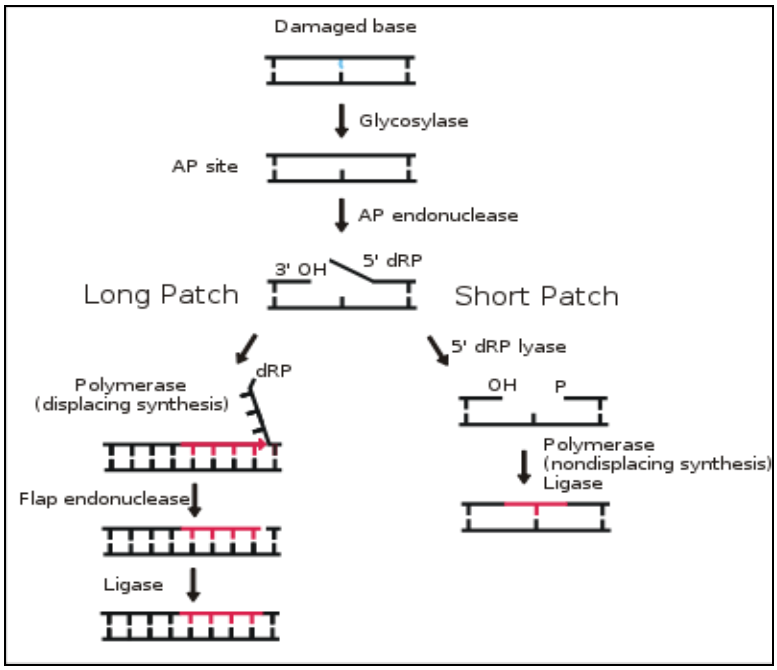


Fig 4. Base excision repair mechanism

Frequently occurring hydrolysis reaction is the deamination of cytosine to uracil and, adenine to hypoxanthine. Basic enzymes involved in BER and their actions are as follows:

DNA GLYCOSYLASES:

Seven different genes for DNA glycosylases have been identified in *E. coli.* Enzymatic base excision was first observed for uracil. Uracil accumulates in the genome at a rate of around 100 lesions per cell per day (for a genome size of 3 × 109bp).Since this lesion is directly mutagenic therfore all living organisms probably produce a uracil glycosylase (Seeberg *et al.*, 1995). The uracil DNA glycosylase is specifically involved in the repair of uracil-containing DNA, but it was recently shown that the enzyme can also remove 5-hydroxyuracil. The enzyme is extremely well conserved from bacteria to humans (56% identity), indicating the essential nature of this type of function in preventing mutations arising from deaminated cytosine residues in DNA.

3 METHYLADENINE GLYCOSYLASE:

This enzyme has been reported in bacteria, yeast, mammals and *Arabidopsis* and shows a varying degree of substrate specificity. 3-Methyladenine is a non-coding lesion .It occurs spontaneously at a significant rate like uracil (Britt and Filippov., 1996). *E. coli* have two 3-methyladenineglycosylases for repair of alkylation damage. The product of the *tag* gene is highly specific for 3-methyladenine, the major cytotoxic alkylation product in DNA while the product of the *alkA* gene comprises only about 10% of the glycosylase activity in cells growing under normal conditions but may be induced 10-fold when cells are exposed to sublethal doses of alkylation. The *alkA* gene has broad substrate specificity, cleaving quantitatively the important alkylation product 7-methylguanine, in addition to several minor but important products. It also has some ability to remove the deamination product hypoxanthine.

UV- ENDONUCLEASES:

Along with several DNA glycosylases, certain organisms contain enzymes popularly known as UV-endonucleases because they produce strand breaks at the site of the pyrimidine dimers. UV-endonucleases cleave the *N*-glycosidic bond of the 5'pyrimidine of the dimer followed by AP-lyase-mediated strand cleavage. The structure of this enzyme has been depicted by X-ray crystallographic analysis and the reaction mechanism has been demonstrated from the structure and site-directed mutagenesis experiments. These enzymes are normally present exclusively in UV resistant organisms, such as *Micrococcus luteus*. However, a similar enzyme has also been coded by the *denV* gene of the bacteriophage T4 and such activity has been detected in *S. cerevisiae*. In addition to the above enzymes a number of other glycosylases and endonucleases have been identified, such as fapy/8-oxoguanine DNA glycosylase endonuclease III/thymine glycol DNA glycosylase, endonuclease VIII,A-G-mismatch DNA glycosylases,5-hydroxymethyl and 5-formyl-uracil DNA glycosylases, along with their possible substrates. (Malta *et al.*, 2008*).*

2.3.2.2 NUCLEOTIDE EXCISION REPAIR

Nucleotide excision repair (NER) is the most flexible and versatile DNA repair pathway of living cells because it deals with a wide range of structurally unrelated DNA lesions. NER corrects a wide array of DNA lesions that distort the DNA double helix, interfere in base pairing and block DNA duplication and transcription. The most common examples of these lesions are the cyclobutane pyrimidine dimers and 6-4 photoproducts induced by ultraviolet radiation and bases with large substitutes derived from chemicals such as polycyclic aromatic hydrocarbons. NER can also correct smaller modified bases. Bacterial nucleotide excision repair was first observed in *E. coli*, by the discovery that UV radiation resulted in the repair synthesis of short stretches of DNA, indicating that it was not just the damaged base that was removed but complete nucleotide

stretches. NER can be subdivided into two subpathways, slow, global genome repair (GGR) and fast, transcription-coupled repair (TCR).

Global repair is the process by which most lesions are repaired regardless of their location in the complete genome. Transcription-coupled repair is characterized by the more rapid repair of lesions in the transcribed strand of an expressed gene than in the non transcribed strand. The mechanism of these two pathways is mainly similar except for damage recognition and for initiation of the process. In *E. coli*, both GGR and TCR require the full set of NER proteins, but transcription-coupled repair additionally requires an actively transcribing RNA polymerase (RNAP) and one additional factor, the transcription repair coupling factor (TRCF), encoded by the *mfd* gene (Abenmacher., 2006). The TRCF is thought to recruit UVR proteins to RNAP arrested at a lesion on the transcribed strand, resulting in rapid repair of the transcription-blocking lesion. NER in *E. coli* requires six proteins: UVRA, UVRB, UVRC, UVRD, DNA polymerase I, and ligase .UvrA is present both as a monomer and a dimer, the latter complexing with UVRB for initial DNA damage recognition. This $UVRA_2B$ heterotrimer carry out limited, ATP-dependent, processive scanning of the damaged region until the actual damage site is found. At this point, conformational change occurs in the protein-DNA complex, leading to release of the UVRA dimer, stable UVRB-DNA binding and a local bending and unwinding of the damaged region of DNA. UvrC then binds to the UvrB-DNA complex, unmasking the endonuclease activity of UvrB (Crowley and Hanawalt., 1998).

In the case of UV photoproducts, this activity causes an incision to be made four bases 3" away from the lesion. A second incision is made by the UvrBC complex seven bases away 5" from the lesion. UvrD, commonly known as DNA helicase II, releases UvrC and the oligonucleotide between the dual incisions, leaving UvrB at a 12-base gap on one strand. DNA

polymerase I fill this gap and dissociate the UvrB protein from the DNA. The repair process is completed by DNA ligase, which seals the nick (Fig 5).

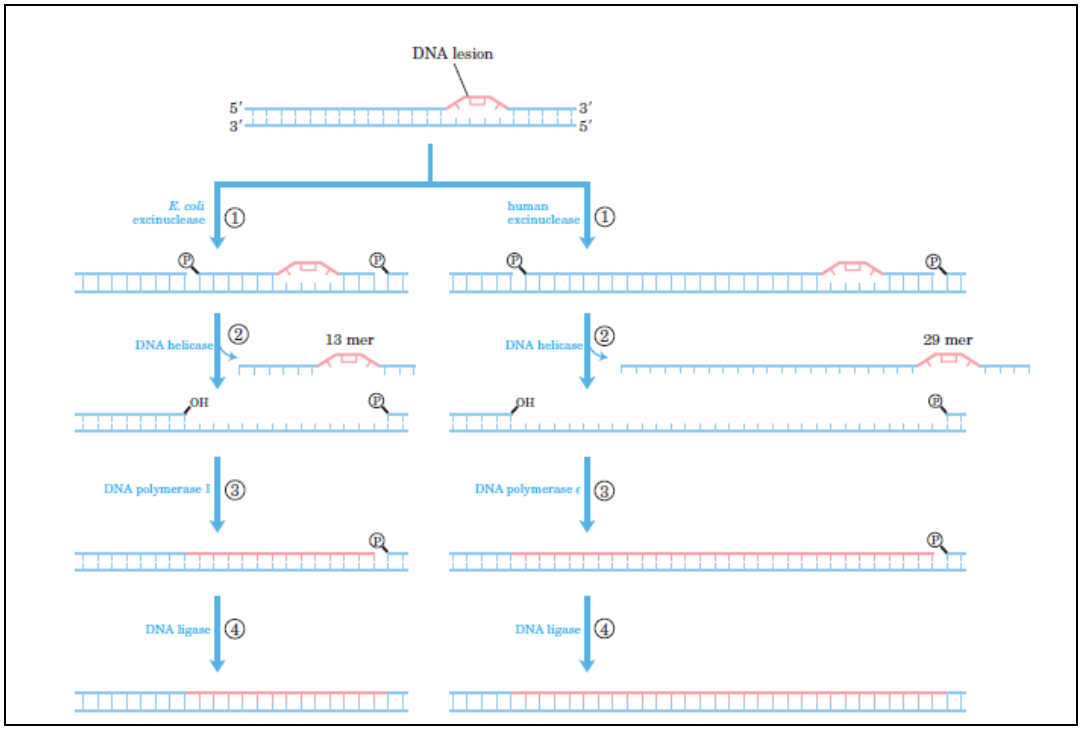


Fig 5. Nucleotide excision repair mechanism

2.3.3 RECOMBINATION REPAIR

Recombination is one of the most important processes involved in DNA repair, ensuring the transmission of correct genetic information from parents to their offspring. Two different modes of recombination in bacteria are

1) *Homologous recombination*, which utilize large regions of DNA homology, usually the homological chromosome, to exchange damaged DNA for the intact one

2) *Non-homologous recombination* in which double-strand breaks (DSBs) of different chromatin regions are joined together on the basis of microhomologies to produce a new gene configuration. However in bacteria the main repair mode is homologous recombination, while in mammals both systems are used by the cell.

2.3.3.1HOMOLOGOUS RECOMBINATION

Recombination is a series of complex biochemical reactions which can repair Double-strand breaks (DSBs) and single-strand gaps in damaged DNA .At least 25 different proteins are involved in all types of homologous recombination in *E. coli* which include the RecA, RecBCD, RecF, RecG, RecJ, RecN, RecO, RecQ, RecR, RuvAB, RuvC, PriA and SSB proteins, DNA polymerases, DNA topoisomerases and DNA ligase, Many of these proteins have functional homologs in other bacteria, eukaryotes and archea. RecA-like protein is present in all free-living organisms. The yeast *Saccharomyces cerevisiae* is an ideal model organism for studying these repair processes. Purified RecA protein can homologously pair and exchange DNA strands *in vitro*. One of the most useful model systems, the three-strand DNA exchange reaction, utilizes circular single-stranded DNA (ssDNA) and homologous linear duplex DNA substrates.

In the presence of a nucleotide cofactor RecA protein polymerizes on the ssDNA to form a helical nucleoprotein filament.This nucleoprotein filament both aligns and pairs with a homologous region in the duplex DNA to form joint molecules. Consequently, the RecA protein filament must accommodate two DNA molecules and must bring them sufficiently close together to promote exchange of DNA strands. DSBs induced directly by ionizing radiation, and indirectly as a natural consequence of DNA replication on a chemically flawed template, are lethal and need repair via recombination pathway. The recombinational repair process consists of four steps: initiation, homologous pairing and DNA strand exchange, DNA heteroduplex extension (branch migration), and Resolution .In the first step of initiation, the RecA protein invades the linear duplex DNA at the DSB to produce the ssDNA. SsDNA formation is a prerequisite for the prototypic homologous pairing reactions promoted by RecA protein. For the second step of recombination, DNA strand exchange occurs between two homologous dsDNA

molecules, processing of one duplex to produce a region of ssDNA is conventionally invoked. This processing involves the recombination-specific helicases, the RecBCD and RecQ proteins,and the latter thought to work in conjunction with the RecJ exonuclease. RecBCD enzyme is a DNA helicase that also possesses a 3”→5” nuclease activity on the strand with the χ sequence, as well as stimulation of the 5” to 3”-nuclease activity responsible for degrading the opposite strand of the duplex. RecBCD is regulated by the direct interaction with the recombination hotspot χ.

In *E. coli* recombination hotspot, known as Chi sites (χ = 5”-GCTGGTGG-3”) enhance the frequency of recombination in their vicinity upto 5-10-fold. Modification of RecBCD enzymatic activity (ssDNAexonuclease, ssDNA endonuclease, ds DNA exonuclease, and DNA dependent ATP-ase and DNA helicase) by χ is coordinated with the loading of RecA protein onto the χ-containing ssDNA by RecBCD enzyme, ensuring incorporation of this ssDNA into a recombinationally proficient nucleoprotein complex. RecQ protein is also a DNA helicase; in wild-type cells it functions in the so-called RecF pathway, which can also act efficiently at DSBs when the RecBCD is rendered non-functional by mutation. If RecA protein fails to assemble on the ssDNA produced, then accessory proteins RecF, RecO and RecR facilitate this assembly step. Upon assembly of a contiguous RecA protein filament on ssDNA, called the presynaptic filament, subsequent homology search can ensue. The third step of recombination is DNA heteroduplex extension; here, a specialized motor protein complex, the RuvAB complex, functions (Kowalczykowski and Bianco., 2000). The RuvAB complex is a DNA helicase that extends the region of DNA heteroduplex by branch migrating the crossover point (Fig 6). The final step of recombination requires separation of the two DNA molecules. This important resolution step is left to a Holliday junction-specific endonuclease, the RuvC protein. The RuvC

protein, as part of a complex with the RuvAB proteins, recognizes and cleaves Holliday junctions to complete the recombination process (Kuzminov and Stahl., 1999).

2.3.5.2 NON HOMOLOGUS END JOINING

Non-homologous end joining (NHEJ) is a pathway which repairs double-strand breaks in DNA. The term "non-homologous end joining" was coined in 1996 by Moore and Haber. NHEJ is referred to as "non-homologous" because the break ends are directly ligated without the need for a homologous template, in contrast to homologous recombination, which requires a homologous sequence to guide the repair mechanism. NHEJ typically utilizes short homologous DNA sequences called microhomologies. These microhomologies are often present in single-stranded overhangs on the ends of double-strand breaks. When the overhangs are perfectly compatible, the breaks are repaired accurately by NHEJ. Imprecise repair leading to loss of nucleotides can also occur, but is much more common when the overhangs are not compatible. NHEJ is evolutionarily conserved throughout all kingdoms from bacteria to mammals and is the predominant double-strand break repair pathway in mammalian cells. NHEJ was originally discovered in eukaryotes and was only identified in prokaryotes in the last decade. Many species of bacteria, including *E.coli*, lack an end joining pathway and thus rely completely on homologous recombination to repair DSBs.

NHEJ proteins have been identified in a number of bacteria, however, including *Bacillus subtilis*, *Mycobacterium tuberculosis*, and *Mycobacterium smegmatis*. Bacteria utilize a remarkably compact version of NHEJ in which all of the required activities are contained in only two proteins: a Ku homodimer and the multifunctional ligase/polymerase/nuclease LigD. In mycobacteria, NHEJ is much more error prone than in yeast, with bases often added to and deleted from the ends of double-strand breaks during repair. Many of the bacteria that possess

NHEJ proteins spend a significant portion of their life cycle in a stationary haploid phase, in which a template for recombination is not available (Zaitsev and Kowalczykowski., 1999).

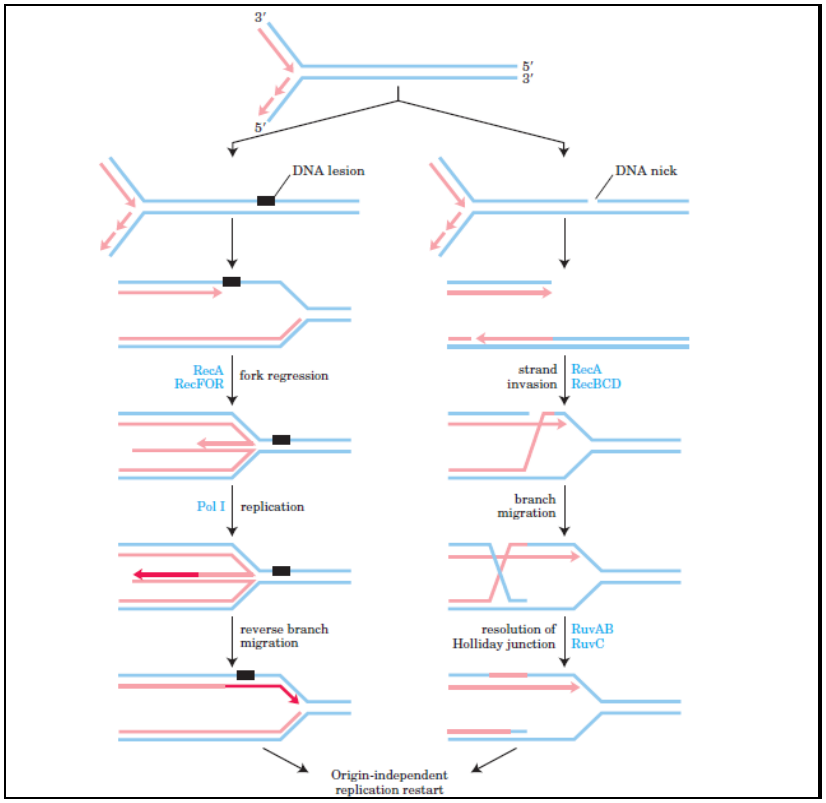


Fig 6. Recombination repair mechanism

2.3.4 MISMATCH REPAIR

DNA mismatch-repair system is involved in the repair of mispaired bases formed during replication, genetic recombination as a result of DNA damage. DNA lesions may be recognized and repaired by more than one DNA-repair process. If two repair systems with different error frequencies have overlapping lesion specificity and one or both is inducible, the resulting variable competition for the lesions can change the biological consequences of these lesions. This concept was demonstrated by observing mutation in *Saccharomyces cerevisiae* exposed to combinations of mutagens under conditions which influenced the induction of error-free

recombinational repair or error-prone repair (Joux *et al.,* 1999). Studies in yeast indicate the involvement of the mismatch repair pathway in prevention of genotoxic effect of oxidative DNA damage. Bacterial cells use differential methylation of the two strands in order to differentiate between "right" or "wrong" undamaged normal base in each strand. *E. coli* MMR system consists of three genes: mutH, mutL, mut S. The first step is the detection of a mismatch through its binding by MutS. MutS recruits MutL and together they activate MutH.

The communication between MutS and MutH was mediated by MutL in an ATP hydrolysis-dependent manner. No biological activity for the MutL protein has been identified, but it may act as "molecular matchmaker", coupling mismatch recognition by MutS to MutH. MutH cleaves the newly replicated error-containing daugther strand at a transiently unmethylated d (GATC) site (Plotz *et al.,* 2003). The degraded strand is replaced by DNA polymerase III holoenzyme which is assisted by single-strand binding proteins, with repair being completed by ligation of the resulting nick by DNA ligase I (Fig 7).

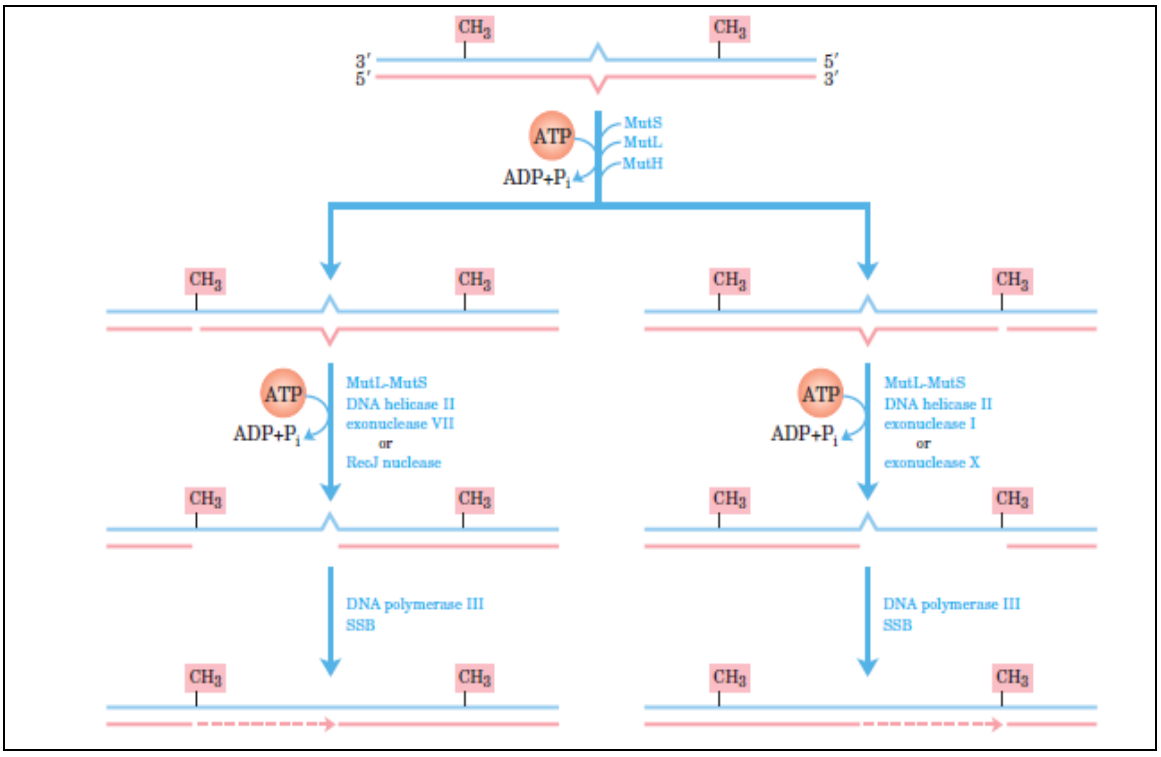


Fig 7. Mismatch Repair Mechanism

2.3.5 SOS REPAIR

The SOS response is a state of high-activity DNA repair. It is activated by bacteria that are exposed to heavy doses of DNA-damaging agents. Their DNA is chopped to shreds, and the bacteria attempts to repair its genome at any cost .The SOS system is a type of regulation mechanism which controls expression of several genes distributed throughout the genome simultaneously (Radman., 1975). The primary control for the SOS regulon is the gene product of *lexA*, which serves as a repressor for *rec.*, *lexA* regulates its own expression along with 16 other proteins that make up the SOS response (Fernandez *et al.*, 2000). During a normal cell's life, the SOS system is turned off, because *lexA* represses expression of all the critical proteins. But when DNA damage occurs, RecA binds to single-stranded DNA. As DNA damage accumulates, more RecA will be bound to the DNA to repair the damage. RecA, in addition to its abilities in recombination repair, stimulates the autoproteolysis of *lexA*'s gene product. That is, LexA will cleave itself in the presence of bound RecA, which causes cellular levels of LexA to drop, which, in turn, causes induction of the SOS regulon genes (Fig 8). As damage is repaired, RecA releases DNA; in this unbound form, it no longer causes the autoproteolysis of LexA, and so the cellular levels of LexA rise to normal again, shutting down expression of the SOS regulon genes.

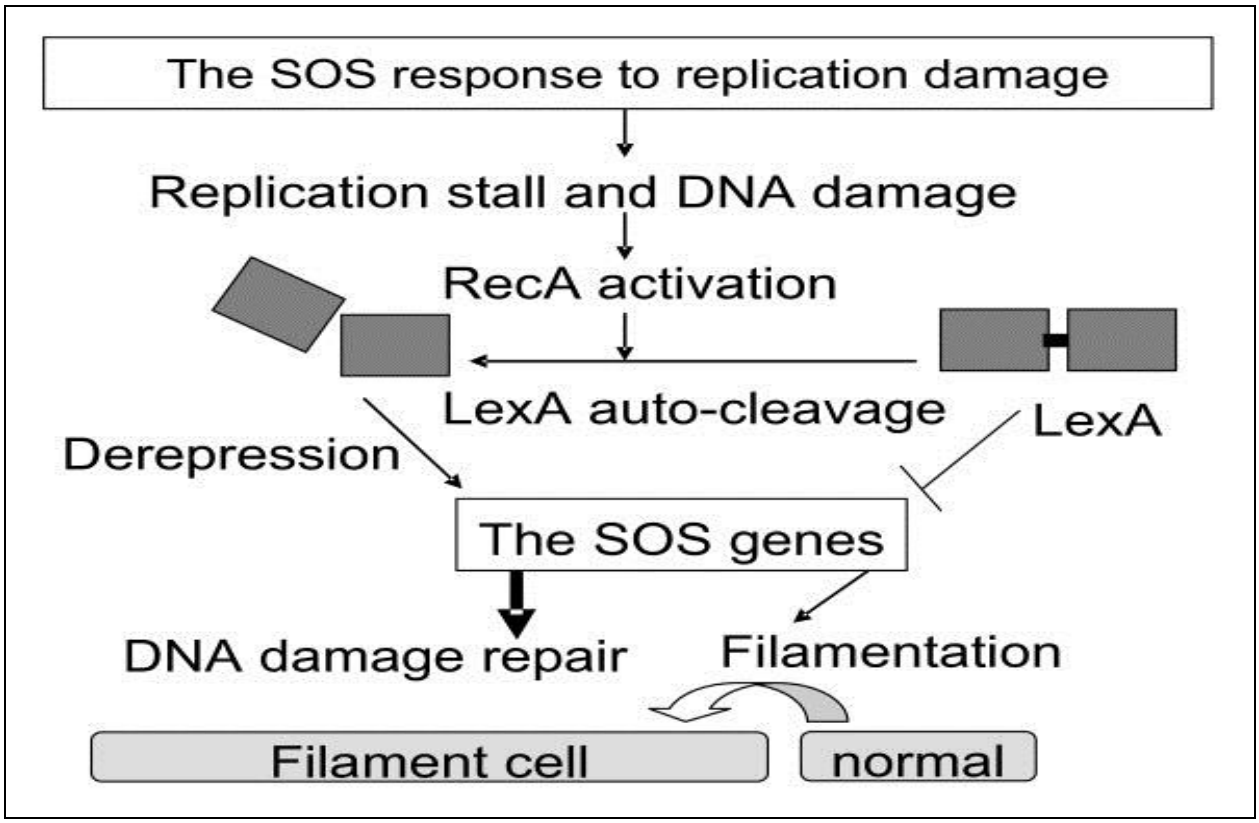


Fig 8. SOS Repair Mechanism

2.4 EFFECT OF pH CHANGE ON MARINE BACTERIA

With the advent of the industrial age the oceans have acquired up one-quarter to one-third of anthropogenic CO_2 emissions. It already led to a reduction in surface ocean pH to 0.1 units, which may reach up to 0.7 units assuming the depletion of all fossil fuel reserves during the next three centuries. In contrast, pH has constantly remained above 8.1 for the last 23 million years (Palmer and Pearson., 2000). The effects of the anticipated rapid reduction in pH on marine organisms, and their ability to adapt, will determine future marine biodiversity and ecosystem functions. But the impact of ocean acidification on different groups of marine organisms remains under debate especially heterotrophic bacteria as they play major roles in marine biogeochemical cycles. It was recently argued that microbe-dependent processes will not substantially change in a more acidic ocean, as marine microbes have already experienced large regional, temporal and

depth-dependent pH variability, and even greater pH ranges are observed in freshwater lakes (Joint *et al.*, 2011). Changes in pH are likely to have a physiological effect, as bacteria living in the alkaline marine water with pH values generally above 8.0 have to invert energy into the homeostasis of their cytoplasmic pH (7.4–7.8). As a result if the difference between external and internal pH becomes smaller due to ocean acidification, bacteria may profit energetically, depending on their pH homoeostasis mechanisms. Nitrogen fixing bacteria also respond to changes in pH, these changes may be a secondary effect on ocean acidification or a more direct result from climate-driven changes in circulation or stratification. Elevated CO_2 may stimulate the production of DOM which will lead to higher heterotrophic bacterial production and respiration and in turn to more material being processed by the rest of the microbial loop.

2.5 EFFECT OF CHANGE OF NaCl CONCENTRATION IN MARINE BACTERIA:

Salinity is defined as the total amount of solid material in grams contained in 1 kg of seawater when all the carbonate ions has been converted to oxide, the bromine and iodine replaced by chlorine, and all organic matter completely oxidized. Normal seawater has a salinity of 35% though the salinity of most of the ocean is rather constant there are areas where salinity undergoes wide fluctuations, especially in near-shore environments where seawater is diluted due to runoff from the land. Dilute seawater refers to water having a salinity of less than 35% (Stanley and Morita., 1968). For bacteria with salt tolerance, growth and salt concentration have a direct correlation. As the amount of salt in the growth medium increases, bacterial growth decreases, for bacteria that require salt, a bell curve of growth is observed. Salinity of the growth medium is also found to have a marked effect on the maximal growth temperature of the marine bacteria. A decrease in the maximal growth temperature was observed at both low and high concentrations of NaCl. The maximum growth temperature, optimal growth temperature, and the

estimated normal physiological range for growth are all the functions of water activity (a_w), which can be manipulated by changing the concentration of sodium chloride (Nichols *et al.*, 2000). It has been found that chloride ions of the sodium chloride is essential for growth at high salt Na^+ concentration in various species of bacteria. It is essential for bacteria to measure external osmolarity and thus they adjust their osmolarity according to their metabolism. Na^+ ion is toxic and every living organism have a tendency to expel out these ions from their cytoplasm.Transport is mediated by Na^+/H^+ antiporters which may be salt induced and efficiency of Na+ export might be enhanced by simultaneous export of Cl via the ClC-type sodium channels found in bacteria (Muller *et al.*, 2007).

3. OBJECTIVES

Based upon the literature surveyed the current work was planned with the following objectives:

- To study the tolerance of marine bacteria on UV exposure.
- To study the effect of light on survival of marine bacteria after UV exposure.
- To find the effect of salt on survival of marine bacteria upon UV exposure.
- To find the effect of pH on survival of marine bacteria upon UV exposure.
- To study the DNA damage in marine bacteria.

4. MATERIALS AND METHODS

4.1 CHEMICALS REQUIRED

*Nutrient Agar Medium (per litre):*0.5% peptone, 0.3% beef extract, 1.5 % Agar,0.5 %NaCl, pH 7).

Luria Bertani Broth (per litre): Tryptone -10g Yeast Extract-5g, NaCl-10g.

TE buffer: 1M Tris buffer, 0.5 M EDTA solution.

TAE buffer: 40mM Tris, 20mM acetic acid, and 1mM EDTA.

Lysis buffer: 2.5 M NaCl, 100mM EDTA, 10 mM TRIS, 1% sodium laurylsarcosine and 1% Triton X-100 at pH 10.

Enzyme Digestion Solution: 2.5M NaCl, 10mM EDTA, 10 mM TRIS,0.5-0.6mg/ml of Proteinase K at pH 7.4.

Electrophoresis Buffer: 300mM sodium acetate and 100 mM TRIS at pH 9.

Neutralisation Buffer: 1M ammonium acetate prepared in ethanol.

PBS solution: NaCl- 8.0g/l, KCl- 0.2g/l, Na_2HPO_4- 1.44g/l, KH_2PO_4- 0.24g/l.

4.2 BACTERIAL CULTURE

Bacterial isolates *Psuedomonas pseudoalcaligenes* NP103 and *Psuedomonas aeruginosa* N6P6 are used for studying the DNA damage and repair.

4.3 THE TOTAL VIABLE COUNT OF THE BACTERIA

A 0.5 ml of each cell suspension was taken in order to prepare serial dilutions in 0.9% NaCl. Samples (50 µl) of the appropriate serial dilutions were spread on Nutrient agar petriplates at an interval of 0,2,4,6,8,12,16,20 and 24 h. Plates were kept in an incubator for overnight at 37◦C and colony were counted to find the total number of viable cells.

4.4 TOLERANCE OF MARINE BACTERIA ON UVR EXPOSURE

4 ml of each culture was centrifuged (6000 rpm, 10 min) to collect the pellet. It was resuspended in 10 ml saline and poured in autoclaved petriplates. Then it was exposed to UV for 5 sec, 10 sec, 15 sec, 20 sec and 25 sec.2 µl of each UV exposed culture was transferred to well of microtiter plate containing 200 µl of LB medium and Optical density (OD) was measured at 595nm wavelength in ELISA plate reader and growth pattern is observed after 24 h.

4.5 TO STUDY THE EFFECT OF LIGHT ON SURVIVAL OF MARINE BACTERIA ON UVR EXPOSURE

Samples (50 µl) of the appropriate serial dilutions 10^{-6} (control) and 10^{-3} were spread on duplicate nutrient agar petri dishes. Plates were exposed to UV for 5 sec, 10 sec, 15 sec, 20 sec and 30 sec.1 plate of each exposure time was kept in light and 1 in dark for 24 h incubation at 37◦C and number of CFU was determined.

4.6 EFFECT OF NaCl CONCENTRATION ON SURVIVAL OF MARINE BACTERIA ON UVR EXPOSURE:

4 ml of each culture was centrifuged(6000 rpm,10 min) to collect the pellet, resuspended in 10 ml saline and exposed to UV for 5 sec, 10 sec,15 sec, 20 sec and 25 sec. 2 µl of UV exposed culture were transferred to microtiter well containing LB of different salt concentration(6%, 3.5%, 2.25% ,1.75% and 1 %) .Optical density (OD) was measured at 595nm and growth pattern was observed after 24 h of incubation at 37◦C.

4.7 EFFECT OF pH ON SURVIVAL OF MARINE BACTERIA ON UVR EXPOSURE

4 ml of each culture was centrifuged (6000 rpm, 10 min) to collect the pellet, resuspended in 10 ml saline and exposed to UV for 5sec, 10sec, 15 sec, 20 sec and 25 sec. 2 µl of UV exposed culture were transferred to microtiter well containing LB of different pH (6, 7, 8, 9).Optical density (OD) was measured at 595 nm and growth pattern was observed after 24 h of incubation at 37^{o}C.

4.8 AGAROSE ASSAY FOR DNA DAMAGE

2 ml of each was centrifuged (6000 rpm, 10 min) to obtain the pellets. The pellets were resuspended in 6 ml of saline and exposed to UV for 0 sec, 10sec and 15 sec. 2 ml of each exposed material was centrifuged (6000 rpm, 10 min). Pellet was resuspended in 567 µl of TE buffer.30 µl of 10% SDS and 3µl of 20mg/ml of proteinase K was added thoroughly mixed and kept in incubation for 1h at 37ºC.After 1h 80 µl NaCl was added and mixed thoroughly.1 volume(0.7-0.8 ml) of 24:1 chloroform – isoamyl alcohol was added and centrifuged (6000 rpm , 15 min) at 4ºC The supernatant was transferred to fresh tubes and 1 volume of 25:24:1 phenol-chloroform-isoamylalcohal was mixed and centrifuged (6000 rpm , 15 min) at 4◦C.The supernatant was again transferred to fresh tubes.0.6 volume of isopropanol was added and mixed

gently to form a stingy white DNA precipitate. It was again centrifuged (10,000 rpm, 10 min) the pellet was washed with 70% cold ethanol, vacuum dried, and dissolved in 50 µl TE. The sample was run on 1% agarose gel to get the damaged DNA bands.

4.9 COMET ASSAY FOR DNA DAMAGE:

Prepartion of culture:

2 ml of fresh cultures was centrifuged (6000 rpm , 10 min).Pellets were resuspended in1 ml PBS and centrifuged (6000 rpm for,10 min).Pellet was again mixed with 2ml of PBS solution.and exposed to UV for 10 sec and 30 sec and centrifuged (6000 rpm, 10 min) and pellet was resuspended in 200 µl of PBS solution.

Slide preparation:

6 slides were prepared by briefly dripping in 1% agarose solution (0.25 g agarose in 25ml of PBS solution). Precoated slides were then dried in incubation at 60◦C-70◦C for 15 min.

Microgel formation and Processing

2 µl of exposed cells were mixed with 200µl of 0.5% Low melting agarose and mixed thoroughly.100 µl of this was poured symmetrically on the prepared slide to form the 2^{nd} layer.A 3^{rd} layer (0.5% low melting agarose,5µg/ml RNAse A,1mg/ml lysozyme and 0.25% N-lauroylsarcosine)was then made over the 2^{nd} layer .Slides were refrigerated for 10 min at 4◦C and incubated for 30 min at 37°C.Slides were then lysed by immersing in a lysis solution containing 2.5 M NaCl,100 mM EDTA,10mM TRIS,1% sodium laurylsarcosine and 1% Triton X-100 at pH 10.After lysis slides were immersed in an enzyme digestion solution(2.5M NaCl,10mM EDTA,10mM TRIS,0.5-0.6mg/ml of Proteinase K at pH 7.4) for 2 h at 37°C.

Electrophoresis and Slide processing

Slides were transferred to an electrophoresis buffer (300mM sodium acetate and 100 mM TRIS at pH 9) for 50 minutes. After electrophoresis slides were immersed in 1M ammonium acetate prepared in ethanol for 20 min. It was then immersed in absolute ethanol for 30 min and then in 70% ethanol for 10 min.

Staining and Visualisation

Prior to staining slides were preheated with a freshly prepared solution of 5% DMSO solution and 10 mM NaH_2PO_4.While the slides were wet DNA was stained with 50 µl of Propidium iodide stain and comets were observed under Fluoroscent Microscope.

5. RESULT

5.1 GROWTH CURVE: TOTAL VIABLE COUNT OF BACTERIA

The growth of *P. psuedoalcaligenes* NP103 and *P. aeruginosa* N6P6 was monitored in terms of OD_{595} and viable cell count for 24 h (Table 5.1). In both the strains the stationary phase was observed after 12 h of growth (Fig. 5.1).

TABLE 5.1 OD_{595} and CFU/ml of P. *psuedoalcaligenes* NP103 and P. *aeruginosa* N6P6 at different time intervals.

Time interval	**P.*psuedoalcaligens* NP103**		**P. *aeruginosa* N6P6**	
	A_{595}	**CFU/ml**	**A_{595}**	**CFU/ml**
0 h	0.008	2.4×10^5	0.006	6.4×10^5
1 h	0.205	3.6×10^5	0.200	1.12×10^6
2 h	0.258	6×10^6	0.232	2.84×10^7
4 h	0.399	1.4×10^7	0.311	4.96×10^7
6 h	0.469	2.24×10^8	0.402	6.48×10^8
8 h	0.488	4.22×10^8	0.416	8.96×10^8
12 h	0.562	4.8×10^8	0.432	1.22×10^{10}
16 h	0.691	6.36×10^9	0.542	1.44×10^{10}
20 h	0.692	1.13×10^{10}	0.684	1.6×10^{10}
24 h	1.124	1.35×10^{10}	1.024	1.76×10^{10}

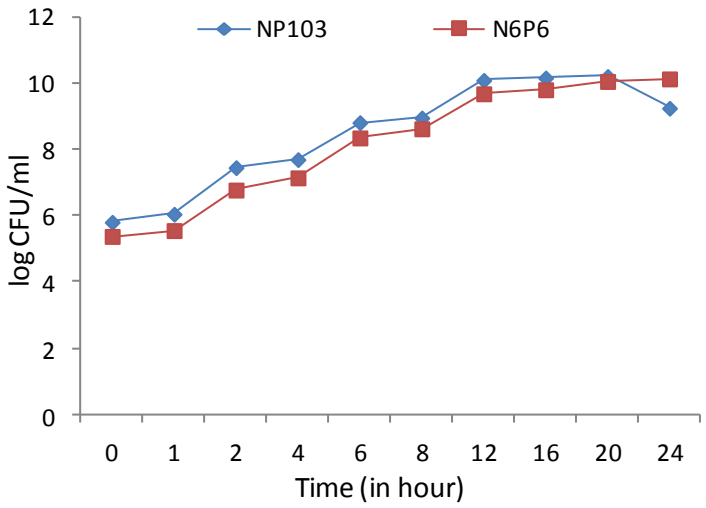


Fig 5.1. Growth cuve for *P. psuedoalcaligenes* NP103 and *P. aeruginosa* N6P6

5.2 TOLERANCE OF MARINE BACTERIA TO UVR EXPOSURE

The effect of UVR-B exposure on the growth of *P. psuedoalcaligenes* NP103 and *P. aeruginosa* N6P6 was monitored spectrophotometrically (Table 5.2). The growth expressed as OD_{595} upon UV exposure after 24 h is shown in Fig 5.2. In both the strains a decrease in growth with increase in exposure time to UV was observed.

Table 5.2 Effect of UVR exposure on growth of *P. psuedoalcaligenes* NP103 and *P. aeruginosa* N6P6. Data are expressed as mean±SD of OD_{595} after 24 h.

UV exposure time	Absorbance at 595 nm		CFU/ml	
	NP103	N6P6	NP103	N6P6
Control(0 sec)	1.124±0.26	0.748±0.01	-	-
5 sec	0.679±0.21	0.621±0.02	2824×10^4	352×10^5
10 sec	0.582±0.26	0.521±0.04	2144×10^6	2424×10^4
15 sec	0.5±0.24	0.441±0.04	2×10^6	32×10^5
20 sec	0.331±0.12	0.39±0.04	56×10^4	464×10^4
25 sec	0.241±0.04	0.09 ±0.04	64×10^4	152×10^4

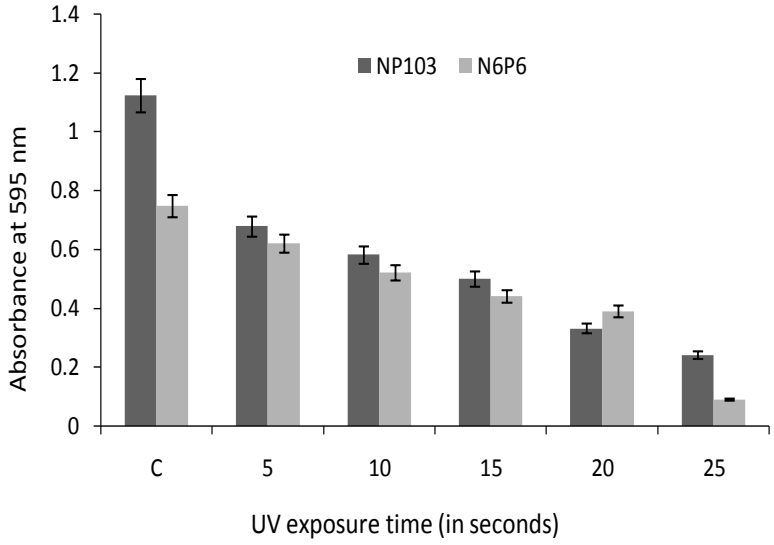


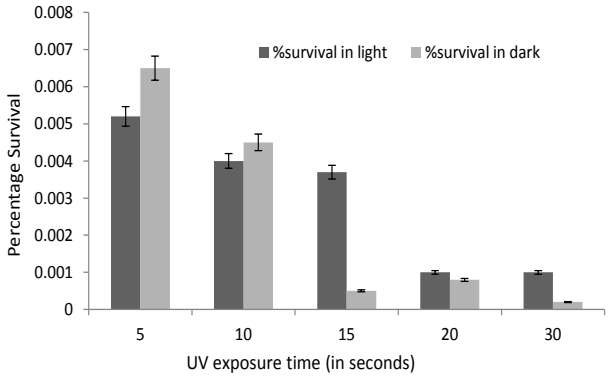
Fig 5.2 Growth at different UV exposure time for *P. psuedoalcaligenes* NP103 and *P. aeruginosa* N6P6

5.3 EFFECT OF LIGHT ON SURVIVAL OF MARINE BACTERIA UPON UVR EXPOSURE

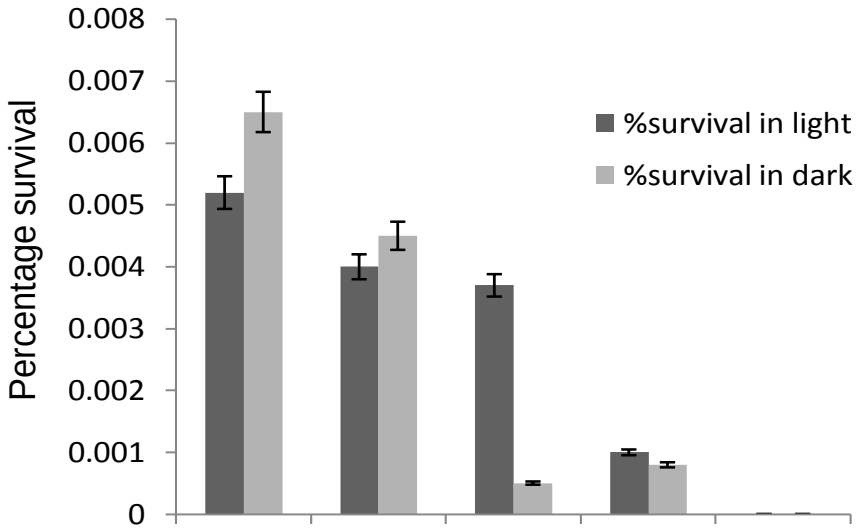
The percentage survival was calculated by viable count method for both *P. pseudoalcaligens* NP103 and *P. aeruginosa* N6P6 The survival is found to be 100% without UVR exposure but on exposure to UVR for different time,the survival percentage decreased (Table 5.3). The percentage survival for *P. pseudoalcaligens* is 0.005 % in 5 sec and it is found to be 0.004% for *P. aeruginosa* (Fig 5.3).

Table 5.3: Percentage survival on UV exposure for *P. pseudoalcaligens* NP103 and *P. aeruginosa* N6P6

UV exposure time	**% survival in NP103**		**% survival in N6P6**	
	Light	Dark	Light	Dark
0 sec	100	100	100	100
5 sec	0.0052	0.0065	0.004	0.0045
10 sec	0.004	0.0045	0.0037	0.0005
15 sec	0.0037	0.0005	0.001	0.0008
20 sec	0.001	0.0008	0	0
30 sec	0.001	0.0002	0	0



(a)



(b)

Fig 5.3 (a) Effect of light in *P. psuedoalcaligenes* NP103 **(b)** Effect of light in *P. aeruginosa* N6P6

5.4 EFFECT OF pH ON SURVIVAL OF MARINE BACTERIA ON UVR EXPOSURE

The effect of UVR exposure on the growth of *P. psuedoalcaligenes* NP103 and *P. aeruginosa* N6P6 was monitored spectrophotometrically (Table 5.4) (Table 5.5).After UVR exposure growth was monitored at different pH. The growth of *P. psuedoalcaligenes* NP103 was maximum at pH 8 (Fig 5.4) on UVR exposure and growth of *P. aeruginosa* N6P6 was optimum at pH 7 (Fig 5.5) on UVR exposure. Thus it is seen that though both the bacterial genus are same their response to pH differs from species to species.

Table 5.4 Effect of pH on growth of *P. psuedoalcaligens* upon UVR exposure after 24 h of incubation. Data are expressed as mean±SD of OD_{595}

UV exposure time	**pH**			
	6	**7**	**8**	**9**
Control	2.3895±0.54	2.398±0.44	2.673±0.44	2.408±0.44
5 sec	2.109±0.54	2.22±0.44	2.17±0.44	2.068±0.44
10 sec	1.3365±0.54	1.561±0.44	1.791±0.44	1.5325±0.44

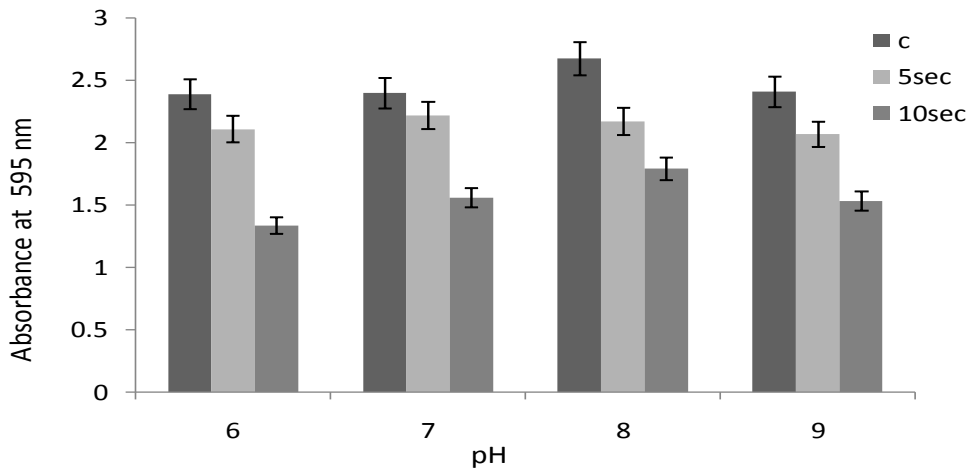


Fig 5.4 Growth at different pH for *P. pseudoalcaligenes* NP103

Table 5.5 Effect of pH on growth of *P. aeruginosa* upon UVR exposure after 24 h of incubation.

Data are expressed as mean±SD of OD_{595}

UV exposure time	**pH**			
	6	**7**	**8**	**9**
Control	1.87±0.05	5.25±0.02	1.141±0.04	0.832±0.11
5 sec	1.784±0.05	5.292±0.02	1.117±0.04	0.768±0.11
10 sec	1.776±0.05	5.262±0.02	1.054±0.04	0.617±0.11

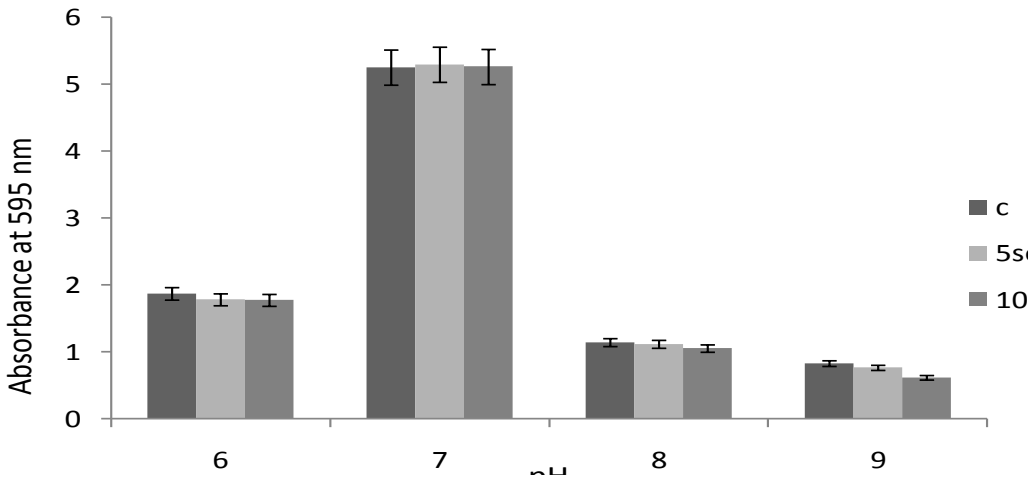


Fig 5.5 Growth at different pH for *P. aeruginosa* N6P6

5.5 EFFECT OF NaCl CONCENTRATION ON SURVIVAL OF MARINE BACTERIA ON UVR EXPOSURE:

The effect of UVR exposure on the growth of *P. psuedoalcaligenes* NP103 and *P. aeruginosa* N6P6 was monitored spectrophotometrically (Table 5.6 and 5.7) After UVR exposure growth was monitored at different NaCl concentration. The growth of *P. psuedoalcaligenes* NP103 was maximum at 1.75% salt on UVR exposure (Fig 5.6) and growth of *P. aeruginosa* N6P6 was maximum at 3.5% salt on UVR exposure (Fig 5.7).

Table 5.6 Effect of NaCl concentration on growth of *P.psuedoalcaligens* NP103 upon UVR exposure after 24 h of incubation. Data are expressed as mean±SD of OD_{595}

UV exposure time	**NaCl concentration**				
	6 %	**3.5%**	**2.25%**	**1.75%**	**1%**
Control	0.428±0.16	1.51±0.57	2.009±0.50	2.077±0.29	0.58±0.18
5sec	0.204±0.16	1.18±0.57	1.85±0.50	2.10±0.29	0.69±0.18
10 sec	0.116±0.16	0.39±0.57	1.07±0.50	1.57±0.29	0.33±0.18

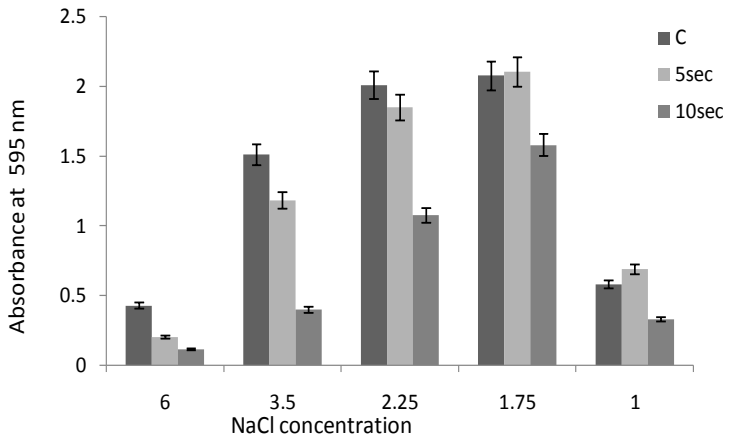


Fig 5.6. Growth at different NaCl concentration for *P. psuedoalcaligens* NP103

Table 5.7 Effect of NaCl concentration on growth of *P. aeruginosa* upon UVR exposure after 24 h of incubation. Data are expressed as mean±SD of OD_{595}

UV exposure time	**NaCl concentration**				
	6 %	**3.5%**	**2.25%**	**1.75%**	**1%**
Control	1.016±0.30	2.866±1.03	2.22±0.36	1.943±0.59	0.748±0.19
5sec	0.668±0.30	1.242±1.03	2.024±0.36	0.98±0.59	0.803±0.19
10 sec	0.4±0.30	1.034±1.03	1.507±0.36	0.86±0.59	0.441±0.19

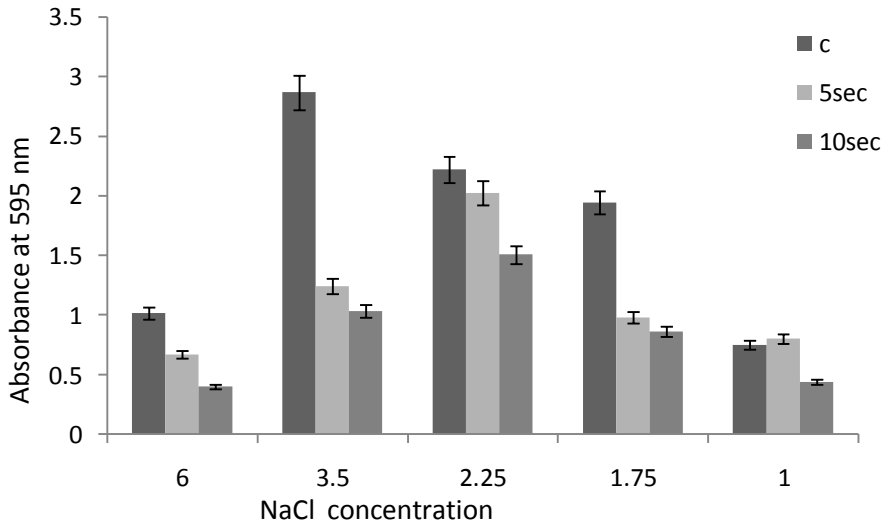
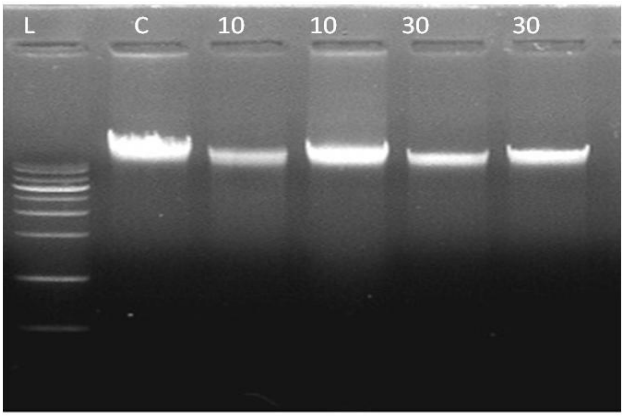


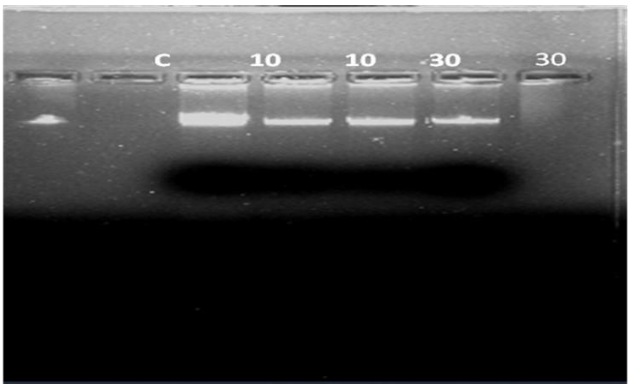
Fig 5.7 Growth at different concentration of NaCl for *P. aeruginosa* N6P6

5.6 AGAROSE ASSAY FOR DNA DAMAGE DETECTION:

In the agarose assay, DNA extracted from UVR exposed cells were run on the agarose gel for both *P. psuedoalcaligenes* and *P.aeruginosa.* In both the strains of bacteria an intact band was observed in the control well. On exposure to UVR instead of bands smear should be formed, but clear smears were not visible (Fig 5.8) so the damage is further diagnosed and validated by other DNA damage detection methods.



(a) L-ladder of 100 bp; C-control; 10-10sec UV; 30-30sec UV



(b) C-control; 10-10sec UV; 30-30sec UV

Fig 5.8 (a) Gel image for *P. psuedoalcaligenes* NP103 **(b)** Gel image for *P. aeruginosa* N6P6

5.7 COMET ASSAY FOR DNA DAMAGE DETECTION:

Comet assay is done and slides were observed under 40X magnification of fluorescent microscope. A round head like structure is found in control of both *P. psuedoalcaligens* NP103 and *P. aeruginosa* N6P6 (Fig 5.9). The tail size increased from 10 sec of UV exposure to 30 min of UV exposure in both the strains of bacteria (Fig 5.10). It can thus be inferred that with an increase in UVR exposure there is damage to the bacterial DNA.

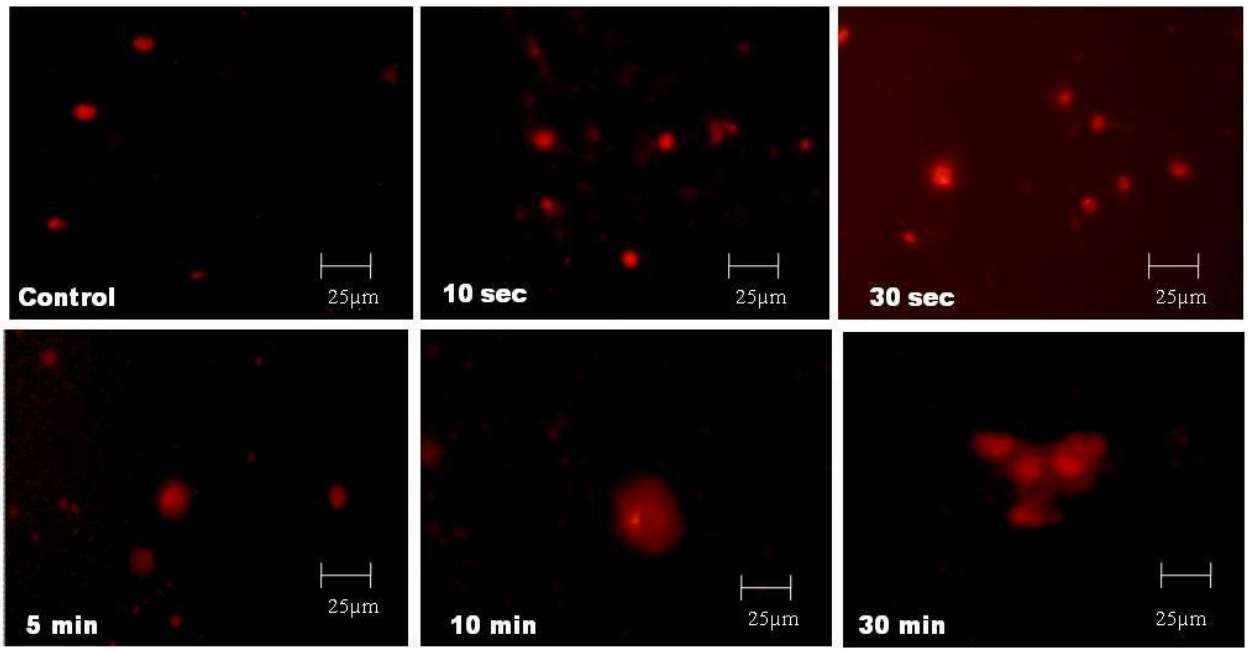


Fig 5.9. Comet results of *P. psuedoalcaligens* NP103 for control and 10 sec, 30 sec, 5 min,10 min and 30 min of UV exposure.

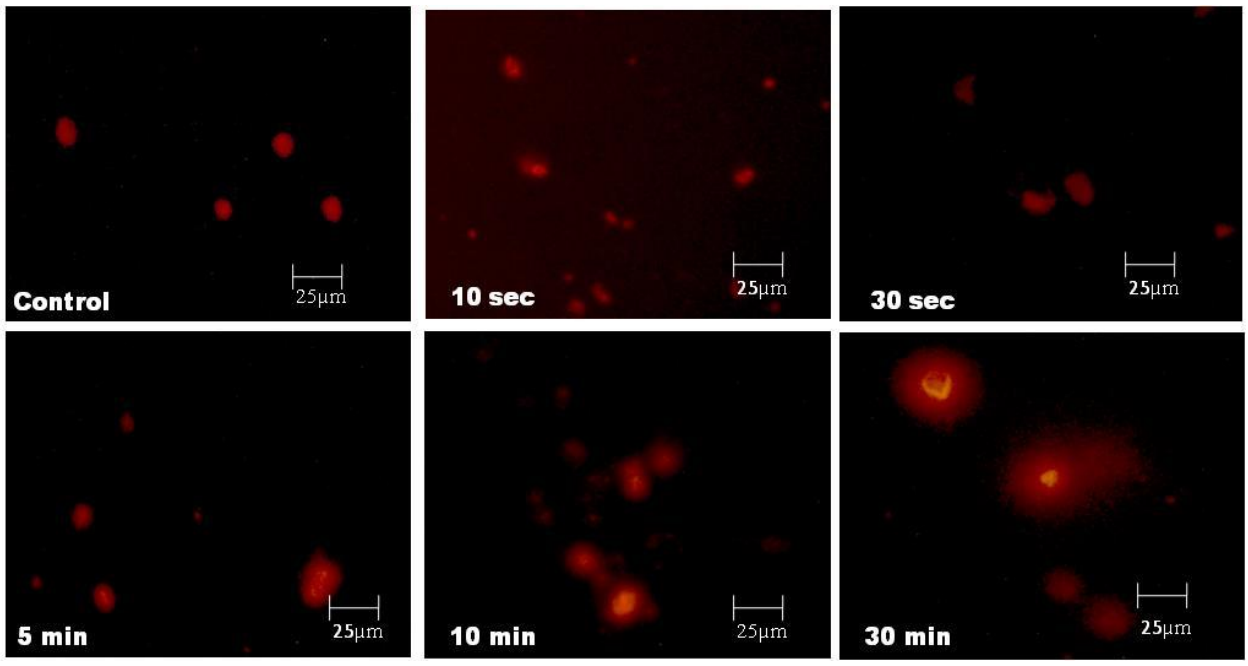


Fig 5.10 Comet results of *P. aeruginosa* N6P6 for control and 10 sec, 30 sec, 5 min, 10 min and 30 min of UV exposure.

6. DISCUSSION

The presence of UV-damage specific repair genes lost and gained in the several bacterial species as a response to a change in habitat or DNA damaging agents. Photolyase is the oldest UV damage repair protein which must have evolved in the first organisms that inhabited regions where they were exposed to UV light. The NER proteins evolved later, after branching of the archaea from the eubacteria. The NER system has the advantage over the photolyases that it can repair UV lesions in the dark. We studied the response of *Psuedomonas* to the UV light spectrophotometrically. It is found that the both *P. psuedoalcaligenes* NP103 and *P. aeruginosa* N6P6 showed reduction in growth in response to UVR exposure time. The percentage survival on exposure to light is also studied. It is seen that initially dark repair mechanism was more active and with passage of time photorepair also becomes significant.

Increase in the emission of CO_2 to the atmosphere has cause significant increase in temperature (global warming) and decline in ocean water pH (ocean acidification). This change also plays an important role in functioning of marine bacteria. A comprehensive study on the effect of change of pH and salt concentration is also carried out. Sea water pH is typically limited to a range of 7.5 – 8.4 .It is found that pH 8 is the best pH for bacterial growth in marine environment a further increase in pH decreased the growth rate in the bacteria (Krause *et al.*, 2012). Growth of *P. psuedoalcaligenes* NP103 after UVR exposure was highest at pH 8 whereas for *P. aeruginosa* N6P6 optimum growth after UVR exposure was found at pH 7.

The salinity of marine environment also alters in response to global warming. In the present study, with the decrease in NaCl concentration up to certain level cause increase in growth of the bacteria. Growth is maximum at a NaCl concentration of 3.5% in *P. aeruginosa*

N6P6 whereas in *P. pseudoalcaligenes* NP103 it is found to be 1.75% which is in accordance with (Stanley and Morita., 1968).

All the bacteria have a damage response mechanism to increase their chance of survival in response to the UV radiation and other damage causing agents. Several methods are used to find out the amount of damage in the bacterial cells. Here we have used Comet Assay and Agarose Assay to find the amount of damage caused. The tail is formed in UV exposed slide and Heads are seen in control slides in the Comet Assay which reflects that with an increase in UV exposure damage increases (Haydel and Solanky., 2012).

Thus, from the above study we can say that UV exposure plays an important role in bacterial growth pattern along with change in salt concentration and pH activity, but in response to these damages bacterial species have developed their enhanced mode of repair mechanism as a result of their survival strategy.

7. CONCLUSIONS

In the era of Global warming, ozone depletion, ocean acidification and their effect on survival of aquatic organisms are the rising problem and accepted fact. Oceanic uptake of anthropogenic carbon dioxide (CO_2) is altering the seawater chemistry of the world's oceans with consequences upon marine microbes. UV acts as an additional stressor on these microbes thereby altering the major biogeochemical cycles of the nature. DNA is certainly one of the key targets for UV-induced damage in a variety of organisms ranging from bacteria to humans as UV radiation induces abundant mutagenic and cytotoxic DNA lesions .As bacteria are believed to have originated in the Precambrian era at a time when the ozone shield was absent, they must have faced high fluxes of UV radiation,that must have acted as an evolutionary pressure leading to the selection for efficient UV radiation protecting and repair mechanisms. The influence of UV-B at the ecosystem level is more pronounced on community and trophic level structure, and hence on subsequent biogeochemical cycles In our study we not only studied the harmful effect of UV on bacteria but also the effect of change in NaCl concentration and oceanic pH due to increase in atmospheric CO_2.We also studied the DNA damage detection techniques. It provides a nutshell idea on the effect of harmful UV radiation on the microbes of marine environment. It also provides scope to study further about the lethal aspects of DNA damage by other advanced techniques. Thus, we can conclude that global warming and ocean acidification will not only cause harm to microbial communities but have subsequent effect on organisms of higher trophic level and microbial processes in the ocean.

8. REFERENCES

Abenmacher, N. (2006). Structural and biochemical analysis of the UvrA binding module of the bacterial transcription repair coupling factor Mfd. *(Doctoral dissertation, lmu)*. 7, 44-62.

Aubert, C., Vos, M. H., Mathis, P., Eker, A. P., Brettel, K. (2000).Intraprotein radical transfer during photoactivation of DNA photolyase. *Nature*, 405(6786), 586-590.

Azam, F., Malfatti, F. (2007). Microbial structuring of marine ecosystems. *Nature Reviews Microbiology*, 5(10), 782-791.

Bertrand, C., Ypersele, J.P.V. (2002). Transient climate simulation forced by natural and anthropogenic climate forcings. *International Journal of climatology*, 22(6), 623-648.

Britt, A.B., Filippov, D.V. (1996). DNA damage and repair in plants. *Annual review of plant biology*, 47(1), 75-100.

Crowley, D.J., Hanawalt, P.C. (1998). Induction of the SOS Response Increases the Efficiency of Global Nucleotide Excision Repair of Cyclobutane Pyrimidine Dimers, but Not 6-4 Photoproducts, in UV-Irradiated Escherichia coli. *Journal of Bacteriology*, 180(13), 3345-3352.

Dizdaroglu, M., Vos, M.H. (2005). Base-excision repair of oxidative DNA damage by DNA glycosylases. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 591(1), 45-59.

Fernandez de Henestrosa, A.R., Ogi, T., Aoyagi, S., Chafin, D., Hayes, J.J., Ohmori, H., Woodgate, R. (2000). Identification of additional genes belonging to the LexA regulon in Escherichia coli. *Molecular microbiology*, 35(6), 1560-1572.

Malta, E., Verhagen, C. P., Moolenaar, G. F., Filippov, D. V., van der Marel, G. A., Goosen, N., (2008). Functions of base flipping in *E. coli* nucleotide excision repair. *DNA repair*, 7(10), 1647-1658.

Haydel, S. E., Solanky, D. (2012). Adaptation of the neutral bacterial comet assay to assess antimicrobial-mediated DNA double-strand breaks in *Escherichia coli*. *Journal of microbiological methods*, *91*(2), 257-261.

Joint, I., Doney, S.C., Karl, D.M. (2011). Will ocean acidification affect marine microbes & quest. *The ISME journal*, 5(1), 1-7.

Joux, F., Mitchel, D.L., Jeffrey, W.H., Lebaron, P. (1999). Marine bacterial isolates display diverse responses to UV-B radiation. *Applied and environmental microbiology*, 65(9), 3820-3827.

Kowalczykowski, S.C., Bianco, P.R. (2000). Translocation step size and mechanism of the RecBC DNA helicase. *Nature*, 405(6784), 368-372.

Krause, E., Wichels, A., Giménez, L., Lunau, M., Schilhabel, M.B., Gerdts, G. (2012). Small changes in pH have direct effects on marine bacterial community composition: a microcosm approach. *PloS one*, 7(10), e47035.

Rastogi, R.P., Richa, Kumar, A., Tyagi, M.B., Sinha, R.P. (2010). Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. *Journal of Nucleic acids*, 90, 163-169.

Kuzminov, A., Stahl, F.W. (1999). Double-strand end repair via the RecBC pathway in Escherichia coli primes DNA replication. *Genes & development*, 13(3), 345-356.

Muller, V., Sewald, X., Saum, S.H., Palm, P., Pfeiffer, F., Oesterhelt, D. (2007). Autoinducer-2-producing protein LuxS, a novel salt-and chloride-induced protein in the moderately halophilic bacterium Halobacillushalophilus. *Applied and environmental microbiology*, 73(2), 371-379.

Nichols, D.S., Olley, J., Garda, H., Brenner, R.R., McMeekin, T.A. (2000). Effect of temperature and salinity stress on growth and lipid composition of *Shewanella gelidimarina*. *Applied and environmental microbiology*, 66(6), 2422-2429.

Palmer, M. R., Pearson, P. N. (2000). Atmospheric carbon dioxide concentrations over the past 60 million years. *Nature*, 406(6797), 695-699.

Plotz, G., Raedle, J., Brieger, A., Trojan, J., Zeuzem, S. (2003). N-terminus of hMLH1 confers interaction of hMutLα and hMutLβ with hMutSα. *Nucleic acids research*, 31(12), 3217-3226.

Radman, M. (1975). SOS repair hypothesis: phenomenology of an inducible DNA repair which is accompanied by mutagenesis. In Molecular mechanisms for repair of DNA. *Springer US*, 355-367.

Radman, M. Jones, M., Wagner, R.(1987). Mismatch repair and recombination in E. coli. *Cell*, 50(4), 621-626.

Kumari, S., Rastogi R. P., Singh, K. L., Singh, S. P., Sinha, R. P. (2008). DNA damage: detection strategies. *EXCLI J*, 7, 44-62.

Ravanat, J. L., Douki, T., Cadet, J. (2001). Direct and indirect effects of UV radiation on DNA and its components. *Journal of Photochemistry and Photobiology B: Biology*, 63(1), 88-102.

Seeberg, E., Eide, L., Bjoras, M. (1995).The base excision repair pathway. *Trends in biochemical sciences*, 20(10), 391-397.

Sinha, R.P., Häder, D.P. (2002). UV-induced DNA damage and repair: a review. *Photochemical & Photobiological Sciences*, 1(4), 225-236.

Stanley, S.O., Morita, R.Y. (1968). Salinity effect on the maximal growth temperature of some bacteria isolated from marine environments. *Journal of bacteriology*, 95(1), 169-173.

Sultana, S., Rana, S., Kumar, R., Sharma, R.K. (2010). Radiation-induced biomarkers for the detection and assessment of absorbed radiation doses. *Journal of Pharmacy And Bioallied Sciences*, 2(3), 189.

Thoma, F. Hanawalt, P.C. (1999). Light and dark in chromatin repair: repair of UV-induced DNA lesions by photolyase and nucleotide excision repair. *The EMBO Journal*, 18(23), 6585-6598.

Wallace, J.M., Hartmann, D.L., Limpasuvan, V., Thompson, D. W., Holton, J. R. (2000). Can ozone depletion and global warming interact to produce rapid climate change?. *Proceedings of the National Academy of Sciences*, 97(4), 1412-1417.

Ward, P.D., Brownlee, D. (2003). Rare Earth: Why complex life is uncommon in the Universe. Springer US, 1-335.

Zaitsev, E.N., Kowalczykowski, S.C. (1999). Enhanced monomer–monomer interactions can suppress the recombination deficiency of the recA142 allele. *Molecular microbiology*, 34(1). 1-9.

Zenoff, V.F., Sineriz, F., Farías, M.E. (2006). Diverse responses to UV-B radiation and repair mechanisms of bacteria isolated from high-altitude aquatic environments. *Applied and environmental microbiology*, 72(12), 7857-7863.

Zhou, B.B.S., Elledge, S.J. (2000). The DNA damage response: putting checkpoints in perspective. *Nature*, 408(6811), 433-439.